DESCRIPTION

IDENTIFICATION AND USE OF MOLECULAR MARKERS INDICATING CELLULAR REPROGRAMMING

This application is related to, and claims priority from, provisional U.S. Patent Application No. 60/209,874, filed on June 7, 2000, which is hereby incorporated by reference in its entirety, including all tables, figures, and claims.

INTRODUCTION

The present invention relates in part to identifying and evaluating the molecular events associated with nuclear and cellular reprogramming. More particularly, the invention identifies one or more "expression events" occurring within cells, tissues, embryos, and/or animals that signal developmental competence or lineage-specific development.

BACKGROUND OF THE INVENTION

The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

Researchers have been developing methods for cloning animals over the past two decades. Some reported methods include the steps of (1) isolating a cell, most often an embryonic cell; (2) inserting that cell or a nucleus isolated from the cell into an enucleated oocyte (*e.g.*, the nucleus of the oocyte was previously extracted), and (3) allowing an embryo to develop from the nuclear transfer oocyte *in vivo*. These methods, while useful, are severly limited due to poor efficiencies, as measured by the birth of live animals. In bovines, for example, embryos generated by *in vitro* fertilization techniques result in live births at a 50% or greater efficiency; artificial insemination techniques efficiencies are 90% or better. In contrast, live birth/nuclear transfer efficiencies are about 1% or less. Current methods to assess embryo viability and developmental competence rely on subjective measurements of embryo quality. *See, e.g.*, Overström, 1996,

Theriogenology **45**: 3-16. In the context of nuclear transfer, these methods have proven to be of limited usefulness.

When a nuclear donor cell is inserted into a recipient oocyte, the oocyte environment alters the inserted nucleus in a process referred to as "cellular reprogramming." This reprogramming can result in a developmentally competent nuclear transfer embryo; that is, an embryo able to result in a live birth. The underlying molecular mechanisms of cellular reprogramming remain poorly understood. Researchers have noted that DNA methylation patterns can be altered in the transition to developmental competence (*see, e.g.,* Surani *et al.*, 1990, *Phil. Trans. R. Soc. Lond. B* **326**: 313-327; Monk, 1990, *Phil. Trans. R. Soc. Lond. B* **326**: 299-312; Surani, 1999, *Seminars in Cell and Dev. Biol.* **10**: 273-277); and that certain uridylic acid-rich nuclear RNA molecules and histone subtypes change as cells transition from developmental competence to a more differentiated state (*see, e.g.*, Ray *et al.*, 1997, *Mol. and Cell. Biochem.* **177**: 79-88; Clarke *et al.*, 1998, *Dev. Genet.* **22**: 17-30).

Researchers have also described various gene products that may be related to pluripotence (*i.e.*, the ability of a cell to differentiate into multiple cell lineages) and/or totipotence (*i.e.*, the ability of a cell to differentiate into all the cells of an animal). Some possible examples are the oct-3 and oct-4 genes in mice (*see, e.g.*, Rosner *et al.*, 1990, *Nature* **345**: 686-92; Shimazaki *et al.*, 1993, *EMBO J.* **12**: 4489-4498; Saijoh *et al.*, 1996, *Genes to Cells* **1**: 239-252; Wang and Schultz, 1996, *Biochem. Cell Biol.* **74**: 579-584; Yeom *et al.*, 1996, *Development* **122**: 881-894; Brehm *et al.*, 1997, *Mol. and Cell. Biol.* **17**: 154-162; Brehm *et al.*, *Acta Pathol. Microbiol. et Immunol. Scand.* **106**: 114-126; Pesce *et al.*, 1998, *BioEssays* **20**: 722-732; and Pesce, 1999, *Cells Tissues Organs* **165**: 144-152); and various mouse homeobox genes (*see, e.g.*, Webb *et al.*, 1993, *Genomics* **18**: 464-466; and Chapman *et al.*, 1997, *Genomics* **46**: 223-33).

Moreover, researchers have also attempted to identify gene products that may be related to the ability of a pluripotent cell to differentiate into specific cell lineages, and to isolate specific stem cell populations. *See, e.g.*, Bain *et al.*, 1992, *Soc. Neurosci. Abst.* **18**: 612 (abstract no. 265.13); Bain *et al.*, 1993, *Mol. Brain Res.* **17**: 23-30; Lelias *et al.*,

1993, *Proc. Natl. Acad. Sci. USA* **90**: 1479-1483; Urven *et al.*, 1993, *Biol. Reprod.* **48**: 564-574; U.S. Patent No. 5,639,618, issued on June 17, 1997 to Gay; Hendrikx *et al.*, 1997, *Exper. Hematol.* **25**: 878 (abstract no. 522); Walther and Bader, 1999, *Mol. Brain Res.* **68**: 55-63; and U.S. Patent No. 5,874,301, issued on February 23, 1999 to Keller *et al.*

Additionally, researchers have developed a trap vector approach to identify potential developmentally related or lineage related genes. *See, e.g.*, von Melchner *et al.*, 1992, *Genes and Dev.* **6**: 919-927; Reddy et al., 1992, *Proc. Natl. Acad. Sci. USA* **89**: 6721-6725; Bruyns et al., 1994, *Br. J. Haematol.* **87** (Suppl. 1): 92 (abstract no. 362); Baker et al., 1997, *Dev. Biol.* **185**: 201-214; Muth et al., 1998, *Dev. Dynamics* **212**: 277-283; U.S. Patent No. 5,922,601, issued on July 13, 1999 to Baetscher et al.; and U.S. Patent No. 5,928,888, issued on July 27, 1999 to Whitney.

SUMMARY OF THE INVENTION

The present invention concerns identifying and evaluating the molecular events associated with cellular reprogramming. More particularly, the invention identifies one or more "expression events" occurring within cells, tissues, embryos, and/or animals that signal developmental competence, developmental incompetence, lineage-specific development, viability, totipotency, or pluripotency. These expression events can then be used to efficiently screen and select cells, tissues, embryos, fetuses and/or animals that are competent to undergo reprogramming from amongst a background of incompetent cells, tissues, embryos, fetuses and/or animals. Moreover, methods and molecules able to induce such expression events can be identified and used to induce competence in otherwise incompetent cells, tissues, embryos, fetuses and/or animals.

The materials and methods described herein can be used to increase the efficiencies of cloning by nuclear transfer procedures from a success rate of less than 1% (measured by comparing the number of nuclear transfers required to produce a single live birth) to as much as 50% or more. Among the benefits provided are the ability to optimize culture conditions for competent donor cells and embryos, to optimize oocyte,

donor cell, and embryo handling procedures, and to identify those donor cells, embryos and fetuses most likely to result in a live birth.

Furthermore, the materials and methods described herein can be used to increase the efficiencies of identifying cell populations for use in cell-based therapeutics and tissue regeneration. Among the benefits provided are the ability to optimize culture conditions for inducing stem cell populations to differentiate along a specific selected cell lineage, and to identify those stem cell populations most likely to provide a desired therapeutic benefit.

Thus, in a first aspect, the invention concerns the identification and use of one or more expressed sequence tags, the expression of which can be used to identify a cell, embryo, or fetus as being developmentally competent or developmentally incompetent.

In a first embodiment, cells can be identified as being developmentally competent based on the expression of an expressed sequence tag (or its complementary sequence) known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence. Similarly, cells can be identified as being developmentally incompetent based on the expression of an expressed sequence tag (or its complementary sequence) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent.

The term "expressed sequence tag," or "EST" as used herein refers to an isolated, enriched, or purified nucleic acid sequence representing a gene that is expressed in a tissue-specific or developmentally-specific manner by one or more cells. Such ESTs can be referred to as being "differentially expressed" in two cells or tissues. Preferably, an EST is obtained by sequencing one or more complementary DNA ("cDNA") molecules prepared from messenger RNA ("mRNA") strands present in a specific cell or tissue type. Methods for preparing cDNA molecules are well known to the skilled artisan. *See,*

e.g., Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, NY. In certain embodiments, an EST is obtained from sequences present in a tissue-specific or developmentally-specific cDNA library. An EST may represent the sequence of a full length gene or mRNA molecule, or may contain only a partial sequence. While an EST is preferably a sequence corresponding to an mRNA molecule itself (a "sense" sequence), in certain preferred embodiments an EST can be a sequence that is complementary to a nucleic acid molecule expressed in a tissue-specific or developmentally-specific manner (an "antisense" sequence). The term EST can refer to a sequence in any digital or alphanumeric form, such as a computer file, computer display, or printed table describing each sequence, or to the EST nucleic acid molecules themselves. An EST that is characteristic of a specific cell or tissue type may be referred to as a "marker" of that cell or tissue type.

While the present invention is described in terms of ESTs generated from a cDNA library, the skilled artisan will understand that nucleic acid sequences representing genes that are expressed in a tissue-specific or developmentally-specific manner by one or more cells can also be obtained from genomic DNA sequences. For example, Shoemaker *et al.*, *Nature* 409: 922-927 describes microarray-based methods using exon arrays. Thus, the ESTs of the present invention may come from genomic sources, as well as from mRNA or cDNA sources.

Particularly preferred are one or more ESTs that are markers of developmental competence of cells, developmental incompetence of cells, developmental competence of embryos, developmental incompetence of embryos, lineage-specific development of cells, viability of cells, viability of embryos, viability of fetuses, totipotency of cells, pluripotency of cells, oocyte competence for nuclear transfer, oocyte incompetence for nuclear transfer, oocyte competence for *in vitro* fertilization, and oocyte incompetence for *in vitro* fertilization.

In preferred embodiments, an EST is at least about 9 nucleotides in length, at least about 10 nucleotides in length, at least about 11 nucleotides in length, at least about 12 nucleotides in length, at least about 13 nucleotides in length, at least about 14 nucleotides

in length, at least about 15 nucleotides in length, at least about 16 nucleotides in length, at least about 17 nucleotides in length, at least about 18 nucleotides in length, at least about 19 nucleotides in length, at least about 20 nucleotides in length, at least about 25 nucleotides in length, at least about 30 nucleotides in length, at least about 35 nucleotides in length, at least about 40 nucleotides in length, at least about 45 nucleotides in length, at least about 50 nucleotides in length, at least about 55 nucleotides in length, at least about 60 nucleotides in length, at least about 65 nucleotides in length, at least about 70 nucleotides in length, at least about 75 nucleotides in length, at least about 80 nucleotides in length, at least about 90 nucleotides in length, at least about 100 nucleotides in length, at least about 125 nucleotides in length, at least about 150 nucleotides in length, at least about 175 nucleotides in length, at least about 200 nucleotides in length, at least about 300 nucleotides in length, at least about 400 nucleotides in length, at least about 500 nucleotides in length, at least about 1,000 nucleotides in length, at least about 5,000 nucleotides in length, at least about 10,000 nucleotides in length, at least about 50,000 nucleotides in length, and at least about 100,000 nucleotides in length.

The terms "complementary" and "complement" as used herein in reference to sequences refers to the ability of each of the various nucleotides to form a binding pair by hydrogen bonding with a specific complementary nucleotide. For example, the skilled artisan understands that guanine and cytosine are complementary nucleotides, as are adenine and thymine or uracil. A second sequence is complementary to a first sequence when substantially every nucleotide in the first sequence can be paired in register to a nucleotide in the second sequence. Two nucleic acid strands containing such complementary sequences can "hybridize," or form a double stranded nucleic acid molecule. Nucleic acid hybridization techniques are well known in the art. *See, e.g.*, Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, NY.; U.S. Patent No. 5,935,788, issued on August 10, 1999 to Burmer *et al.*, entitled "Subtractive Hybridization Techniques for Identifying Differentially Expressed and Commonly Expressed Nucleic Acid;" and U.S. Patent No. 5,773,213, issued on June 30, 1998 to Gullans *et al.*, entitled "Method for Conducting Sequential Nucleic Acid Hybridization Steps," each of which is incorporated in its entirety, including all tables, figures, and claims.

In preferred embodiments a second sequence is exactly complementary to a first sequence. In certain embodiments, however, a second sequence may contain one or more mismatched, additional, or deleted nucleotides and still be complementary to a first sequence, so long as the two strands contain sufficient complementary nucleotides for hybridization to occur. In preferred embodiments, a complementary sequence comprises 1% mismatched, additional, or deleted nucleotides, 2% mismatched, additional, or deleted nucleotides, 3% mismatched, additional, or deleted nucleotides, 4% mismatched, additional, or deleted nucleotides, 5% mismatched, additional, or deleted nucleotides, 6% mismatched, additional, or deleted nucleotides, 7% mismatched, additional, or deleted nucleotides, 8% mismatched, additional, or deleted nucleotides, 9% mismatched, additional, or deleted nucleotides, or 10% mismatched, additional, or deleted nucleotides. In particularly preferred embodiments, a sequence can be longer than its complementary sequence due to additional 5' and/or 3' nucleotides that do not overlap with the complementary region.

Isolated nucleic acids are unique in that they are not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally occurring sequence has been removed from its normal cellular (*i.e.,* chromosomal) environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleic acid present, but that it is essentially free (about 90 - 95% pure at least) of non-nucleotide material naturally associated with it, and thus is distinguished from isolated chromosomes.

The term "enriched" in reference to nucleic acids means that the specific DNA or RNA molecule constitutes a significantly higher fraction (2- to 5-fold or more) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other DNA or RNA molecules present, just that the relative amount of the nucleic acid of interest has been significantly increased. The term "significant" is used to

indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2-fold, more preferably at least 5- to 10-fold or even more. The term also does not imply that there is no DNA or RNA from other sources. DNA from other sources may, for example, comprise DNA from a yeast or bacterial genome, or a cloning or expression vector.

It is also advantageous for some purposes that a nucleic acid molecule be in purified form. In this context, "purified" does not require absolute purity (such as a homogeneous preparation). Instead, it represents an indication that the molecule is relatively more pure than in its natural environment (compared to the natural level this level should be at least 2- to 5-fold greater, *e.g.*, in terms of mg/mL). Individual clones isolated from a cDNA library may be purified to electrophoretic homogeneity. cDNA clones are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance, typically messenger RNA (mRNA). The construction of a cDNA library from mRNA involves creating cDNAs by reverse transcription of mRNA. Pure individual cDNA clones can be isolated from the library by clonal selection. Thus, a process that includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximate 10^6-fold purification. Thus, purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

The term "expression" as used herein refers to the presence of an RNA molecule in a cell or tissue as a result of the transcription machinery of the cell or tissue. During transcription in eukaryotic cells, RNA molecules are synthesized from a complementary DNA template by one of three different RNA polymerase molecules. In most cases, the initial transcripted RNA molecule is not a functional RNA molecule, but is instead a precursor molecule that must be processed before it becomes a mature ribosomal, transfer, or messenger RNA molecule. Additionally, both primary transcripts and mature RNA molecules are subject to various degradation enzymes, and thus may be present as fragments of the original full length RNA molecule. The skilled artisan will therefore understand that expression can refer to the presence of the RNA molecule in any of these forms.

The term "developmentally competent" as used herein refers to a cell (or nucleus thereof), embryo, or fetus that is capable of developing into a live born animal. A developmentally competent cell can give rise to all of the cells of an animal when it is utilized as a source of nuclear donor material in a nuclear transfer procedure. In preferred embodiments, a "developmentally competent cell" has not yet been used in a nuclear transfer procedure, but is obtained from a cell line that has been demonstrated to produce cells that are capable of developing into a live born animal. Such a cell line is referred to as a "developmentally competent cell line." A developmentally competent cell can be referred to as "totipotent." A developmentally competent cell may be, but need not be, capable of passing its genetic characteristics through the germ line. In preferred embodiments, a developmentally competent cell line is so identified if 50%, 60%, 70%, 80%, or 90% of nuclear transfer embryos prepared using nuclear donors from that cell line are able to initiate pregnancy and reach 90 days of gestation in a maternal host. In other preferred embodiments, a developmentally competent cell line is so identified if 50%, 60%, 70%, 80%, or 90% of nuclear transfer embryos prepared using nuclear donors from that cell line are able to initiate pregnancy in a maternal host, and 50%, 60%, 70%, 80%, or 90% of those pregnancies result in a live birth.

The term "developmentally competent cell" and "developmentally competent cell line" may also refer to cells and cell lines expressing one or more nucleic acid sequences that are known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence. Such a nucleic acid can be referred to as a "marker" of a developmentally competent cell or cell line.

The term "developmentally incompetent" as used herein refers to a cell (or nucleus thereof), embryo, or fetus that is not capable of developing into a live born animal. In particularly preferred embodiments, a developmentally incompetent cell can give rise to all of the cells of an embryo or fetus when it is utilized as a source of nuclear donor material in a nuclear transfer procedure, but is incapable of giving rise to a live born animal. Thus, a developmentally incompetent cell may be "pluripotent," but is not

"totipotent." In preferred embodiments, a "developmentally incompetent cell" is obtained from a cell line that has been tested for the ability to develop into a live born animal under conditions successfully used with developmentally competent cells, but has failed to demonstrate developmental competence. Such a cell line is referred to as a "developmentally incompetent cell line." In preferred embodiments, a developmentally competent cell line is so identified if less than 50%, less than 40%, less than 30%, less than 20%, or less than 10% of nuclear transfer embryos prepared using nuclear donors from that cell line are able to initiate pregnancy and reach 90 days of gestation in a maternal host.

The term "developmentally incompetent cell" and "developmentally incompetent cell line" may also refer to cells and cell lines expressing one or more nucleic acid sequences that are known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent. Such a nucleic acid can be referred to as a "marker" of a developmentally incompetent cell or cell line.

In preferred embodiments, developmentally competent and incompetent cells include, but are not limited to, cells isolated from an embryo arising from the union of two gametes in vitro or in vivo; embryonic stem cells (ES cells) arising from cultured embryonic cells (*e.g.*, pre-blastocyst cells and inner cell mass cells); inner cell mass cells isolated from of embryos; pre-blastocyst cells; fetal cells; primordial germ cells; germ cells (*e.g.*, embryonic germ cells); somatic cells isolated from an animal; cumulus cells; amniotic cells; fetal fibroblast cells; genital ridge cells; differentiated cells; lineage-specific cells; and totipotent cells.

The term "identifies" or "identifying" as used herein with respect to cells refers to the ability to distinguish between cells having two distinct characteristics. In preferred embodiments, a developmentally competent cell or cell line can be distinguished from a developmentally incompetent cell or cell line. In certain preferred embodiments, an EST or ESTs identify a cell or cell line as "developmentally competent" if the EST sequences

are present and/or expressed in embryos produced by nuclear transfer using a developmentally competent nuclear donor cell, but are present and/or expressed at a reduced or nondetectable level in embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell. Similarly, an EST or ESTs identify a cell or cell line as "developmentally incompetent" if the sequences are present and/or expressed in embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell, but are present and/or expressed at a reduced or nondetectable level in embryos produced by nuclear transfer using a developmentally competent nuclear donor cell.

The term "detectable level" as used herein refers to the ability of a comparison method to detect a nucleic acid molecule. The skilled artisan will understand that different comparison methods will have different sensitivities. For example, an RNA molecule present in low abundance in a cell may be below the detectable level of a hybridization assay due to the hybridization conditions used. Moreover, detection of a protein product by immunological means may not detect RNA molecules present in even moderate abundance. But it is well within the skill level of the ordinarily skilled artisan to determine which comparison methods may be appropriately used in specific circumstances. For example, a developmentally regulated RNA molecule may be present in high abundance in one developmental stage, but present in moderate abundance in a second developmental stage. The two developmental stages may be differentiated by a comparison method in which moderate abundance is below the detectable level, but high abundance detectable.

The term "totipotent" as used herein refers to a cell that gives rise to a live born animal. The term "totipotent" can also refer to a cell that gives rise to all of the cells in a particular animal. A totipotent cell can give rise to all of the cells of an animal when it is utilized in a procedure for developing an embryo from one or more nuclear transfer steps. Totipotent cells may also be used to generate incomplete animals such as those useful for organ harvesting, *e.g.*, having genetic modifications to eliminate growth of an organ or appendage by manipulation of a homeotic gene. A totipotent cell may be, but need not be, capable of passing its genetic characteristics through the germ line.

The term "totipotent" as used herein is to be distinguished from the term "pluripotent." The latter term refers to a cell capable of differentiating into a number of different cell types, but that cannot give rise to all of the cells in a live born animal. The term "totipotent" as used herein is also to be distinguished from the term "chimer" or "chimera." The latter term refers to a developing cell mass, such as an embryo, fetus, or animal, that comprises a sub-group of cells harboring nuclear DNA with a significantly different nucleotide base sequence than the nuclear DNA of other cells in that cell mass.

The term "live born" as used herein preferably refers to an animal that exists *ex utero*. A "live born" animal may be an animal that is alive for at least one second from the time it exits the maternal host. A "live born" animal may not require the circulatory system of an in utero environment for survival. A "live born" animal may be an ambulatory animal. Such animals can include pre- and post-pubertal animals. As discussed previously, a live born animal may lack a portion of what exists in a physiologically normal animal of its kind.

In preferred embodiments, developmentally competent cells and developmentally incompetent cells are cultured; are cultured as cell lines; and are cultured as permanent cell lines.

The term "cultured" as used herein in reference to cells can refer to one or more cells that are undergoing cell division or not undergoing cell division in an in vitro environment. An in vitro environment can be any medium known in the art that is suitable for maintaining cells in vitro, such as suitable liquid media or agar, for example. Specific examples of suitable in vitro environments for cell cultures are described in Culture of Animal Cells: a manual of basic techniques (3rd edition), 1994, R.I. Freshney (ed.), Wiley-Liss, Inc.; Cells: a laboratory manual (vol. 1), 1998, D.L. Spector, R.D. Goldman, L.A. Leinwand (eds.), Cold Spring Harbor Laboratory Press; and Animal Cells: culture and media, 1994, D.C. Darling, S.J. MorganJohn Wiley and Sons, Ltd., each of which is incorporated herein by reference in its entirety including all figures, tables, and drawings. Cells may be cultured in suspension and/or in monolayers with one or more substantially similar cells. Cells may be cultured in suspension and/or in

monolayers with a heterogeneous population of cells. The term "heterogeneous" as utilized in the previous sentence can relate to any cell characteristics, such as cell type and cell cycle stage, for example. Cells may be cultured in suspension, cultured as monolayers attached to a solid support, and/or cultured on a layer of feeder cells, for example. Furthermore, cells may be successfully cultured by plating the cells in conditions where they lack cell to cell contact. Cells cultured as monolayers may be grown to confluence, where such cells will cease actively dividing due to contact inhibition. Preferably, cultured cells undergo cell division and are cultured for at least 5 days, more preferably for at least 10 days or 20 days, and most preferably for at least 30 days. Preferably, a significant number of cultured cells do not terminate while in culture. The terms "terminate" and "significant number are defined" hereafter. Nearly any type of cell can be placed in cell culture conditions. Cultured cells can be utilized to establish a cell line.

The term "cell line" as used herein refers to cultured cells that can be passaged at least one time without terminating. The invention relates to cell lines that can be passaged at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 80, 100, and 200 or more times. Cell passaging is defined hereafter. Examples of cell lines include, but are not limited to, cell lines derived from cells isolated from an embryo arising from the union of two gametes in vitro or in vivo; embryonic stem cell lines (ES cells) arising from cultured embryonic cells (*e.g.*, pre-blastocyst cells and inner cell mass cells); cell lines arising from inner cell mass cells isolated from of embryos; cell lines obtained from pre-blastocyst cells; cell lines obtained from fetal cells; cell lines obtained from primordial germ cells; cell lines obtained from germ cells (*e.g.*, embryonic germ cells); cell lines obtained from somatic cells isolated from an animal; cell lines obtained from cumulus cells; cell lines obtained from amniotic cells; cell lines obtained from fetal fibroblast cells; cell lines obtained from genital ridge cells; cell lines obtained from differentiated cells; lineage-specific cell lines; and cell lines obtained from totipotent cells.

In preferred embodiments (1) a cell or a cell line of the present invention is a mammalian cell or cell line; (2) a mammalian cell or cell line is selected from the group

consisting of canid cells or cell lines, felid cells or cell lines, murid cells or cell lines, leporid cells or cell lines, ursid cells or cell lines, mustelid cells or cell lines, and human and non-human primate cells or cell lines; (3) a mammalian cells or cell lines is an ungulate cells or cell lines; and (4) an ungulate cells or cell lines is selected from the group consisting of suid cells or cell lines, ovid cells or cell lines, equid cells or cell lines, bovid cells or cell lines, caprid cells or cell lines, and cervid cells or cell lines.

The term "mammalian" as used herein refers to any animal of the class *Mammalia*. Preferably, a mammalian cell or cell line is a placental, a monotreme and a marsupial. Most preferably, a mammalian cell or cell line is a bovine, a porcine, and a human and non-human primate. A mammalian cell or cell line can be isolated from any source of mammalian cells including, but not limited to, a mammalian embryo, a mammalian fetus, and a mammalian animal.

The term "canid" as used herein refers to any animal of the family *Canidae*. Preferably, a canid cell or cell line is isolated from a wolf, a jackal, a fox, and a domestic dog.

The term "felid" as used herein refers to any animal of the family *Felidae*. Preferably, a felid cell or cell line is isolated from a lion, a tiger, a leopard, a cheetah, a cougar, and a domestic cat.

The term "murid" as used herein refers to any animal of the family *Muridae*. Preferably, a murid cell or cell line is isolated from a mouse and a rat.

The term "leporid" as used herein refers to any animal of the family *Leporidae*. Preferably, a leporid cell or cell line is isolated from a rabbit.

The term "ursid" as used herein refers to any animal of the family *Ursidae*. Preferably, a ursid cell or cell line is isolated from a bear.

The term "mustelid" as used herein refers to any animal of the family *Mustelidae*. Preferably, a mustelid cell or cell line is isolated from a weasel, a ferret, an otter, a mink, and a skunk.

The term "primate" as used herein refers to any animal of the *Primate* order. Preferably, a primate cell or cell line is isolated from an ape, a monkey, a chimpanzee, and a lemur.

The term "ungulate" as used herein refers to any animal of the polyphyletic group formerly known as the taxon *Ungulata*. Preferably, an ungulate cell or cell line is isolated from a camel, a hippopotamus, a horse, a tapir, and an elephant. Most preferably, an ungulate cell or cell line is isolated from a sheep, a cow, a goat, and a pig.

The term "ovid" as used herein refers to any animal of the family *Ovidae*. Preferably, an ovid cell or cell line is isolated from a sheep.

The term "suid" as used herein refers to any animal of the family *Suidae*. Preferably, a suid cell or cell line is isolated from a pig or a boar.

The term "equid" as used herein refers to any animal of the family *Equidae*. Preferably, an equid cell or cell line is isolated from a zebra or an ass. Most preferably, an equid cell or cell line is isolated from a horse.

The term "bovid" as used herein refers to any animal of the family *Bovidae*. Preferably, an bovid cell or cell line is isolated from an antelope, an oxen, a cow, a bison, and a goat.

The term "caprid" as used herein refers to any animal of the family *Caprinae*. Preferably, an caprid cell or cell line is isolated from a goat.

The term "cervid" as used herein refers to any animal of the family *Cervidae*. Preferably, an cervid cell or cell line is isolated from a deer.

The term "terminating" and "terminate" as used herein with regard to cultured cells may refer to cells that undergo cell death, which can be measured using multiple techniques known to those skilled in the art (*e.g.*, CytoTox96® Cytotoxicity Assay, Promega, Inc. catalog no. G1780; Celltiter96® Aqueous Cell Proliferation Assay Kit, Promega, Inc. catalog no. G3580; and Trypan Blue solution for cytotoxicity assays,

Sigma catalog no. T6146). Termination may also be a result of apoptosis, which can be measured using multiple techniques known to persons skilled in the art (*e.g.*, Dead End™ Apoptosis Detection Kit, Promega, Inc. catalog no. G7130). Terminated cells may be identified as those that have undergone cell death and/or apoptosis and have released from a solid surface in culture. In addition, terminated cells may lack intact membranes which can be identified by procedures described above. Also, terminated cells may exhibit decreased metabolic activity, which may be caused in part by decreased enzymatic activity that can be identified by calcein AM, for example. Furthermore, termination can be refer to cell cultures where a significant number of cultured cells terminate. The term "significant number" in the preceding sentence can refer to about 80% of the cells in culture, preferably about 90% of the cells in culture, more preferably about 100% of the cells in culture, and most preferably 100% of the cells in culture.

The term "suspension" as used herein refers to cell culture conditions in which cells are not attached to a solid support. Cells proliferating in suspension can be stirred while proliferating using apparatus well known to those skilled in the art.

The term "monolayer" as used herein refers to cells that are attached to a solid support while proliferating in suitable culture conditions. A small portion of cells proliferating in a monolayer under suitable growth conditions may be attached to cells in the monolayer but not to the solid support. Preferably less than 15% of these cells are not attached to the solid support, more preferably less than 10% of these cells are not attached to the solid support, and most preferably less than 5% of these cells are not attached to the solid support.

The term "plated" or "plating" as used herein in reference to cells can refer to establishing cell cultures in vitro. For example, cells can be diluted in cell culture media and then added to a cell culture plate, dish, or flask. Cell culture plates are commonly known to a person of ordinary skill in the art. Cells may be plated at a variety of concentrations and/or cell densities.

The term "cell plating" can also extend to the term "cell passaging." Cells of the invention can be passaged using cell culture techniques well known to those skilled in the

art. The term "cell passaging" can refer to a technique that involves the steps of (1) releasing cells from a solid support or substrate and disassociation of these cells, and (2) diluting the cells in media suitable for further cell proliferation. Cell passaging may also refer to removing a portion of liquid medium containing cultured cells and adding liquid medium to the original culture vessel to dilute the cells and allow further cell proliferation. In addition, cells may also be added to a new culture vessel which has been supplemented with medium suitable for further cell proliferation.

The term "proliferation" as used herein in reference to cells can refer to a group of cells that can increase in number over a period of time.

The term "confluence" as used herein refers to a group of cells where a large percentage of cells are physically contacted with at least one other cell in that group. Confluence may also be defined as a group of cells that grow to a maximum cell density in the conditions provided. For example, if a group of cells can proliferate in a monolayer and they are placed in a culture vessel in a suitable growth medium, they are confluent when the monolayer has spread across a significant surface area of the culture vessel. The surface area covered by the cells preferably represents about 50% of the total surface area, more preferably represents about 70% of the total surface area, and most preferably represents about 90% of the total surface area.

In further embodiments, expressed sequence tags can be grouped in numbers of 2 or more, and up to numbers of 10,000 or more, to provide a gene expression database. The expression of one or more expressed sequence tags in the database can be used to identify cells, embryos, or fetuses as being developmentally competent or developmentally incompetent.

Preferably, a gene expression database comprises two or more expressed sequence tags (or their complementary sequences) known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence. As discussed above, such ESTs can be referred to as being "differentially expressed." Cells, embryos, and

fetuses can be identified as developmentally competent based on the presence of at least one of the ESTs in such a gene expression database. In particularly preferred embodiments, a cell, embryo or fetus is identified as developmentally competent based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Likewise, a gene expression database preferably comprises two or more expressed sequence tags (or their complementary sequences) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent. Cells, embryos, and fetuses can be identified as developmentally incompetent based on the presence of at least one of the former ESTs, and the absence of the latter ESTs. In particularly preferred embodiments, a cell, embryo or fetus is identified as developmentally incompetent based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Most preferably, a gene expression database comprises at least one EST (or its complementary sequence) known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence; and at least one EST (or its complementary sequence) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent. In such embodiments, cells, embryos, and fetuses can be identified as developmentally competent based on the presence of at least one of the former ESTs, and the absence of the latter ESTs. Likewise, cells can be identified as

developmentally incompetent based on the presence of at least one of the latter ESTs, and the absence of the former ESTs.

The term "gene expression database" as used herein refers to any set of two or more different ESTs. In certain preferred embodiments, a gene expression database can be a representation of two or more EST sequences in any digital or alphanumeric form, such as a computer file, computer display, or printed table describing each sequence. In other preferred embodiments, a gene expression database can be any format containing the EST nucleic acid molecules themselves. For example, a solution or a solid phase comprising two or more different ESTs can be a gene expression database as that term is used in the instant invention. In preferred embodiments, a gene expression database can contain at least about 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, 25000, 30000, 40000, 50000, or 100,000 different ESTs.

Particularly preferred are gene expression databases that contain one or more markers of developmental competence of cells, developmental incompetence of cells, developmental competence of embryos, developmental incompetence of embryos, lineage-specific development of cells, viability of cells, viability of embryos, totipotency of cells, pluripotency of cells, oocyte competence for nuclear transfer, oocyte incompetence for nuclear transfer, oocyte competence for *in vitro* fertilization, and oocyte incompetence for *in vitro* fertilization.

The term "plurality" as used herein refers to 2 or more. In preferred embodiments, a plurality can be 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, 25000, 30000, 40000, 50000, or 100000 or more.

In yet other embodiments, the invention relates to methods for identifying one or more expressed sequence tags, the expression of which can be used to identify cells, embryos, or fetuses as being developmentally competent or developmentally incompetent.

In preferred embodiments, one or more ESTs are identified by comparing one or more first nucleic acid molecules obtained from one or more embryos produced by

nuclear transfer using a developmentally competent nuclear donor cell to one or more second nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell. ESTs that signal developmental competence are identified as one or more nucleic acid molecules that are present in the population of first nucleic acid molecules, but that are not present at a detectable level in the population of second nucleic acid molecules. Likewise, one or more nucleic acid molecules that are present in the population of second nucleic acid molecules, but that are not present at a detectable level in the population of first nucleic acid molecules are identified as ESTs that signal developmental incompetence.

In particularly preferred embodiments, an EST that signals developmental competence is a nucleic acid molecule (1) present in at least about 75% of embryos produced by nuclear transfer using a developmentally competent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 75% of tested embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell; (2) present in at least about 90% of embryos produced by nuclear transfer using a developmentally competent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 90% of tested embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell; (3) present in at least about 95% of embryos produced by nuclear transfer using a developmentally competent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 95% of tested embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell; and (4) present in at least about 100% of embryos produced by nuclear transfer using a developmentally competent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 100% of tested embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell.

In other particularly preferred embodiments, an EST that signals developmental incompetence is a nucleic acid molecule (1) present in at least about 75% of embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell that

are tested for the presence of the EST, but not present at a detectable level in at least about 75% of tested embryos produced by nuclear transfer using a developmentally competent nuclear donor cell; (2) present in at least about 90% of embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 90% of tested embryos produced by nuclear transfer using a developmentally competent nuclear donor cell; (3) present in at least about 95% of embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 95% of tested embryos produced by nuclear transfer using a developmentally competent nuclear donor cell; and (4) present in at least about 100% of embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell that are tested for the presence of the EST, but not present at a detectable level in at least about 100% of tested embryos produced by nuclear transfer using a developmentally competent nuclear donor cell.

The term "comparing" as used herein in reference to nucleic acid molecules refers to the process of determining the homology or identity of a first nucleic acid sequence to a second nucleic acid sequence. Methods for comparing two nucleic acid sequences are well known to the skilled artisan. In preferred embodiments, such methods can comprise comparing the two sequences in any digital or alphanumeric form, such as a computer file, computer display, or printed table describing each sequence. In this case, comparisons can be made by eye, that is, by a direct comparison by the skilled artisan, or can rely on various computer programs known in the art. *See, e.g.*, Altschul, *et al.* (1997) Nucleic Acids Res. 25:3389-3402 (Gapped BLAST or PSI-BLAST), Altschul, *et al.* (1990) J. Mol. Biol. 215:403-410 (BLAST), and Smith, *et al.* (1981) J. Mol. Biol. 147:195-197 (Smith-Waterman). In other preferred embodiments, comparison methods can comprise comparing two nucleic acid molecules themselves, for example by hybridization methods such as southern blotting, northern blotting, *in situ* hybridization, dot or slot blotting, arrayed nucleic acids (including nucleic acid macroarrays and microarrays, particularly DNA macroarrays and microarrays), and phage display. *See generally,* Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, New York; U.S. Patent No. 6,004,755,

issued on December 21, 1999 to B. Wang, each of which is hereby incorporated in its entirety, including all drawings, claims, and tables. In such methods, typically a nucleic acid molecule that is complementary to a first sequence is compared to a second sequence. In particularly preferred embodiments, two nucleic acid molecules can be compared indirectly by comparing each nucleic acid to a reference nucleic acid library, preferably obtained from the same species as the source of the nucleic acid molecules. The term "nucleic acid library" is defined herein. The foregoing examples are not intended to be exclusive, and other methods for comparing two nucleic acid sequences known in the art are within the scope of the instant invention.

The term "comparing" may also refer to determining the homology or identity of one nucleic acid sequence to another by determining the homology or identity molecules produced from the nucleic acid sequences. For example, mRNA can be used by cells or by *in vitro* translation systems to produce proteins or peptides. These peptides or proteins can be compared by various immunological methods such as immunoblotting, competitive or noncompetitive immunoassay, and immunoprecipitation, and by various nonimmunological methods such as analytical centrifugation, amino acid analysis, sequencing, 1- and 2-dimensional electrophoresis (including both native and denaturing conditions such as SDS-PAGE), chromatography, peptide mapping, nuclear magnetic resonance, electron crystallography, and X-ray crystallography. See generally, Deutscher, ed., 1990, *Methods in Enzymology*, Volume 182, Academic Press, San Diego, CA. Such methods can be referred to by the skilled artisan as "proteomics" or "functional proteomics" techniques.

In preferred embodiments, a nucleic acid sequence or molecule is at least about 9 nucleotides in length, at least about 10 nucleotides in length, at least about 11 nucleotides in length, at least about 12 nucleotides in length, at least about 13 nucleotides in length, at least about 14 nucleotides in length, at least about 15 nucleotides in length, at least about 16 nucleotides in length, at least about 17 nucleotides in length, at least about 18 nucleotides in length, at least about 19 nucleotides in length, at least about 20 nucleotides in length, at least about 25 nucleotides in length, at least about 30 nucleotides in length, at least about 35 nucleotides in length, at least about 40 nucleotides in length, at least about

45 nucleotides in length, at least about 50 nucleotides in length, at least about 55 nucleotides in length, at least about 60 nucleotides in length, at least about 65 nucleotides in length, at least about 70 nucleotides in length, at least about 75 nucleotides in length, at least about 80 nucleotides in length, at least about 90 nucleotides in length, at least about 100 nucleotides in length, at least about 125 nucleotides in length, at least about 150 nucleotides in length, at least about 175 nucleotides in length, at least about 200 nucleotides in length, at least about 300 nucleotides in length, at least about 400 nucleotides in length, at least about 500 nucleotides in length, at least about 750 nucleotides in length, at least about 1000 nucleotides in length, at least about 1250 nucleotides in length, at least about 1500 nucleotides in length, at least about 2000 nucleotides in length, and at least about 3000 nucleotides in length.

The term "homology" as used herein in reference to nucleic acid molecules refers to the amount of sequence similarity between a first and a second nucleic acid molecule. Two molecules displaying sufficient homology are said to be "homologous" to one another. The skilled artisan will understand that a second sequence may contain one or more mismatched, additional, or deleted nucleotides and still be homologous to a first sequence. In preferred embodiments, a homologous sequence comprises 1% mismatched, additional, or deleted nucleotides, 2% mismatched, additional, or deleted nucleotides, 3% mismatched, additional, or deleted nucleotides, 4% mismatched, additional, or deleted nucleotides, 5% mismatched, additional, or deleted nucleotides, 6% mismatched, additional, or deleted nucleotides, 7% mismatched, additional, or deleted nucleotides, 8% mismatched, additional, or deleted nucleotides, 9% mismatched, additional, or deleted nucleotides, or 10% mismatched, additional, or deleted nucleotides. A sequence displaying no mismatched, additional, or deleted nucleotides is said to be "identical" to a first sequence. In particularly preferred embodiments, a sequence can be longer than a homologous or identical sequence due to additional 5' and/or 3' nucleotides that do not overlap with the homologous or identical region.

In certain embodiments, two molecules are referred to as homologous if they contain sufficient sequence identity that a third nucleic acid molecule used as a probe is capable of hybridizing to both molecules. In particularly preferred embodiments, the

probe molecule is complementary to one of the two homologous molecules. The skilled artisan will understand that the amount of homology required between the two molecules such that a probe will bind to both can be variable, depending on the stringency of the hybridization conditions employed.

Homology of two nucleic acid molecules may also be determined from assessing the amount of sequence similarity between a first and a second molecule produced from the nucleic acid sequences. For example, peptides or proteins can be compared by the various methods described above, and homologous nucleic acids identified based on similar or identical peptide maps, amino acid sequences, antibody bindings, *etc.*

The term "identifying" as used herein with respect to nucleic acid molecules refers to selecting one or more molecules exhibiting identity or homology to a target nucleic acid sequence of interest. In preferred embodiments, identifying can refer to selecting sequences representing one or more nucleic acid molecules in any digital or alphanumeric form, such as a computer file, computer display, or printed table describing each sequence. In other preferred embodiments, identifying can comprise selecting one or more nucleic acid molecules themselves.

The terms "nuclear transfer" and "nuclear transfer procedure" as used herein refers to introducing a full complement of nuclear DNA from one cell to an enucleated cell. Nuclear transfer methods are well known to a person of ordinary skill in the art. *See,* U.S. Patent No. 4,994,384 to Prather *et al.*, entitled "Multiplying Bovine Embryos," issued on February 19, 1991; U.S. Patent No. 5,057,420 to Massey, entitled "Bovine Nuclear Transplantation," issued on October 15, 1991; U.S. Patent No. 5,994,619, issued on November 30, 1999 to Stice *et al.*; U.K. Patents Nos. GB 2,318,578 GB 2,331,751, issued on January 19, 2000 to Campbell *et al.* and Wilmut *et al.*, respectively, entitled "Quiescent Cell Populations For Nuclear Transfer"; U.S. Patent No. 6,011,197 to Strelchenko *et al.*, entitled "Method of Cloning Bovines Using Reprogrammed Non-Embryonic Bovine Cells," issued on January 4, 2000; U.S. Patent No. 6,107,543; Proc. Nat'l. Acad. Sci. USA 96: 14984-14989 (1999); Nature Genetics 22: 127-128 (1999); Cell & Dev. Diol 10: 253-258 (1999); Nature Biotechnology 17: 456-461 (1999); Science

289: 1188-1190 (2000); Nature Biotechnol. 18: 1055-1059 (2000); and Nature 407: 86-90 (2000); each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

In a nuclear transfer procedure, a nuclear donor cell, or the nucleus thereof, is introduced into a recipient cell. A recipient cell is preferably an oocyte and is preferably enucleated. However, the invention relates in part to nuclear transfer, where a nucleus of an oocyte is not physically extracted from the nucleus. It is possible to establish a nuclear transfer embryo where nuclear DNA from the donor cell is replicated during cellular divisions. *See, e.g.,* Wagoner *et al.*, 1996, "Functional enucleation of bovine oocytes: effects of centrifugation and ultraviolet light," Theriogenology 46: 279-284. In addition, nuclear transfer may be accomplished by combining one nuclear donor and more than one enucleated oocyte. Also, nuclear transfer may be accomplished by combining one nuclear donor, one or more enucleated oocytes, and the cytoplasm of one or more enucleated oocytes. The resulting combination of a nuclear donor cell and a recipient cell can be referred to variously as a "nuclear transfer embryo," a "hybrid cell," or a "cybrid."

Furthermore, a nuclear donor may arise from an animal of the same specie from which a nuclear recipient is isolated. Alternatively, a nuclear donor may arise from an animal of a different specie from which a nuclear recipient is isolated. For example, a differentiated cell isolated from an ear punch of a water buffalo may be utilized as a nuclear donor and an oocyte isolated from a bovine animal may be utilized as a nuclear acceptor. Thus, xenospecific nuclear transfer is contemplated by the instant invention.

The term "nuclear donor" as used herein refers to any cell having nuclear DNA that can be translocated into an oocyte. A nuclear donor may be a nucleus that has been isolated from a cell. Multiple techniques are available to a person of ordinary skill in the art for isolating a nucleus from a cell and then utilizing the nucleus as a nuclear donor. *See, e.g.*, U.S. Patent No. 4,664,097, which is hereby incorporated by reference in its entirety including all figures, tables and drawings. Any type of cell can serve as a nuclear donor. Examples of nuclear donor cells include, but are not limited to, cultured and non-cultured cells isolated from an embryo arising from the union of two gametes in vitro or

in vivo; embryonic stem cells (ES cells) arising from cultured embryonic cells (*e.g.*, pre-blastocyst cells and inner cell mass cells); cultured and non-cultured cells arising from inner cell mass cells isolated from of embryos; cultured and non-cultured pre-blastocyst cells; cultured and non-cultured fetal cells; cultured and non-cultured primordial germ cells; cultured and non-cultured germ cells (*e.g.*, embryonic germ cells); cultured and non-cultured somatic cells isolated from an animal; cultured and non-cultured cumulus cells; cultured and non-cultured amniotic cells; cultured and non-cultured fetal fibroblast cells; cultured and non-cultured genital ridge cells; cultured and non-cultured differentiated cells; cultured and non-cultured cells in a synchronous population; cultured and non-cultured cells in an asynchronous population; cultured and non-cultured serum-starved cells; cultured and non-cultured permanent cells; and cultured and non-cultured totipotent cells. *See, e.g.*, Piedrahita *et al.*, 1998, *Biol. Reprod. 58*: 1321-1329; Shim *et al.*, 1997, *Biol. Reprod. 57*: 1089-1095; Tsung *et al.*, 1995, *Shih Yen Sheng Wu Hsueh Pao 28*: 173-189; and Wheeler, 1994, *Reprod. Fertil. Dev. 6*: 563-568, each of which is incorporated herein by reference in its entirety including all figures, drawings, and tables. In addition, a nuclear donor may be a cell that was previously frozen or cryopreserved.

In particularly preferred embodiments, a nuclear donor cell is a transgenic cell. The term "transgenic" as used herein in reference to cells refers to a cell whose genome has been altered using recombinant DNA techniques. In preferred embodiments, a transgenic cell comprises one or more exogenous DNA sequences in its genome. In other preferred embodiments, a transgenic cell comprises a genome in which one or more endogenous genes have been deleted, duplicated, activated, or modified. In particularly preferred embodiments, a transgenic cell comprises a genome having both one or more exogenous DNA sequences, and one or more endogenous genes that have been deleted, duplicated, activated, or modified.

The term "activation" refers to any materials and methods useful for stimulating a cell to divide before, during, and after a nuclear transfer step. Cybrids may require stimulation in order to divide after a nuclear transfer has occurred. The invention pertains to any activation materials and methods known to a person of ordinary skill in the art. Although electrical pulses are sometimes sufficient for stimulating activation of cybrids,

other means are sometimes useful or necessary for proper activation of the cybrid. Chemical materials and methods useful for activating embryos are described below in other preferred embodiments of the invention.

Examples of non-electrical means for activation include agents such as ethanol; inositol trisphosphate (IP_3); Ca^{++} ionophores (*e.g.*, ionomycin) and protein kinase inhibitors (*e.g.*, 6-dimethylaminopurine (DMAP)) ; temperature change; protein synthesis inhibitors (*e.g.*, cyclohexamide); phorbol esters such as phorbol 12-myristate 13-acetate (PMA); mechanical techniques; and thapsigargin. The invention includes any activation techniques known in the art. *See, e.g.*, U.S. Patent No. 5,496,720, entitled "Parthenogenic Oocyte Activation" to Susko-Parrish *et al.*, issued on March 5, 1996; and U.S. Patent Application No. 09/176,395, filed on October 21, 1998, each of which is incorporated by reference herein in its entirety, including all figures, tables, and drawings.

The term "fusion" as used herein refers to the combination of portions of lipid membranes corresponding to the totipotent mammalian cell nuclear donor and the recipient oocyte. Lipid membranes can correspond to the plasma membranes of cells or nuclear membranes, for example. The fusion can occur between the nuclear donor and recipient oocyte when they are placed adjacent to one another, or when the nuclear donor is placed in the perivitelline space of the recipient oocyte, for example. Specific examples for translocation of the totipotent mammalian cell into the oocyte are described hereafter in other preferred embodiments. These techniques for translocation are fully described in the references cited previously herein in reference to nuclear transfer.

The term "electrical pulses" as used herein refers to subjecting the nuclear donor and recipient oocyte to electric current. For nuclear transfer, the nuclear donor and recipient oocyte can be aligned between electrodes and subjected to electrical current. The electrical current can be alternating current or direct current. The electrical current can be delivered to cells for a variety of different times as one pulse or as multiple pulses. The cells are typically cultured in a suitable medium for the delivery of electrical pulses. Examples of electrical pulse conditions utilized for nuclear transfer are described in the references and patents previously cited herein in reference to nuclear transfer.

The term "fusion agent" as used herein refers to any compound or biological organism that can increase the probability that portions of plasma membranes from different cells will fuse when a totipotent mammalian cell nuclear donor is placed adjacent to the recipient oocyte. In preferred embodiments fusion agents are selected from the group consisting of polyethylene glycol (PEG), trypsin, dimethylsulfoxide (DMSO), lectins, agglutinin, viruses, and Sendai virus. These examples are not meant to be limiting and other fusion agents known in the art are applicable and included herein.

The term "suitable concentration" as used herein in reference to fusion agents, refers to any concentration of a fusion agent that affords a measurable amount of fusion. Fusion can be measured by multiple techniques well known to a person of ordinary skill in the art, such as by utilizing a light microscope, dyes, and fluorescent lipids, for example.

For the purposes of the present invention, the term "embryo" or "embryonic" as used herein refers to a developing cell mass that has not implanted into the uterine membrane of a maternal host. Hence, the term "embryo" as used herein can refer to a fertilized oocyte, a cybrid (defined herein), a pre-blastocyst stage developing cell mass, and/or any other developing cell mass that is at a stage of development prior to implantation into the uterine membrane of a maternal host. Embryos of the invention may not display a genital ridge. Hence, an "embryonic cell" is isolated from and/or has arisen from an embryo.

An embryo can represent multiple stages of cell development. For example, a one cell embryo can be referred to as a zygote, a solid spherical mass of cells resulting from a cleaved embryo can be referred to as a morula, and an embryo having a blastocoel can be referred to as a blastocyst.

In preferred embodiments (1) an embryo of the present invention is a mammalian embryo; (2) a mammalian embryo is selected from the group consisting of canid embryos, felid embryos, murid embryos, leporid embryos, ursid embryos, mustelid embryos, and human and non-human primate embryos; (3) a mammalian embryos is an ungulate embryo; and (4) an ungulate embryo is selected from the group consisting of

suid embryos, ovid embryos, equid embryos, bovid embryos, caprid embryos, and cervid embryos.

The terms "fetus" and "fetal" as used herein refers to a developing cell mass that has implanted into the uterine membrane of a maternal host. A fetus can include such defining features as a genital ridge, for example. A genital ridge is a feature easily identified by a person of ordinary skill in the art, and is a recognizable feature in fetuses of most animal species. The term "fetal cell" as used herein can refer to any cell isolated from and/or has arisen from a fetus or derived from a fetus. The term "non-fetal cell" is a cell that is not derived or isolated from a fetus.

In preferred embodiments (1) a fetus of the present invention is a mammalian fetus; (2) a mammalian fetus is selected from the group consisting of canid fetuses, felid fetuses, murid fetuses, leporid fetuses, ursid fetuses, mustelid fetuses, and human and non-human primate fetuses; (3) a mammalian fetus is an ungulate fetus; and (4) an ungulate fetus is selected from the group consisting of suid fetuses, ovid fetuses, equid fetuses, bovid fetuses, caprid fetuses, and cervid fetuses.

Additional embodiments relate to methods for preparing gene expression databases comprising two or more, and up to numbers of 10,000 or more, expressed sequence tags, the expression of which can be used to identify cells, embryos, or fetuses as being developmentally competent or developmentally incompetent.

In preferred embodiments, gene expression databases can be prepared by comparing one or more first nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally competent nuclear donor cell to one or more second nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell, and identifying one or more nucleic acid molecules that are present in the population of first nucleic acid molecules, but that are not present at a detectable level in the population of second nucleic acid molecules, to provide two or more expressed sequence tags. ESTs (or their complementary sequences) so identified can then be combined in a gene expression database. Cells, embryos, and fetuses can be identified as developmentally competent

based on the presence of at least one of the ESTs in such a gene expression database. In particularly preferred embodiments, a cell, embryo or fetus is identified as developmentally competent based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Similarly, in other preferred embodiments, gene expression databases can be prepared by comparing one or more first nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell to one or more second nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally competent nuclear donor cell, and identifying one or more nucleic acid molecules that are present in the population of first nucleic acid molecules, but that are not present at a detectable level in the population of second nucleic acid molecules, to provide two or more expressed sequence tags. ESTs (or their complementary sequences) so identified can then be combined in a gene expression database. Cells, embryos, and fetuses can be identified as developmentally incompetent based on the presence of at least one of the ESTs in such a gene expression database. In particularly preferred embodiments, a cell, embryo or fetus is identified as developmentally incompetent based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Most preferably, gene expression databases can be prepared by comparing one or more first nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally competent nuclear donor cell to one or more second nucleic acid molecules obtained from one or more embryos produced by nuclear transfer using a developmentally incompetent nuclear donor cell, and identifying one or more ESTs that are present in the population of first nucleic acid molecules, but that are not present at a detectable level in the population of second nucleic acid molecules, and one or more ESTs that are present in the population of second nucleic acid molecules, but

that are not present at a detectable level in the population of first nucleic acid molecules. ESTs (or their complementary sequences) so identified can then be combined in a gene expression database.

In particularly preferred embodiments, the comparing step comprises comparing one or more nucleic acid molecules to a reference nucleic acid library, preferably obtained from the same species as the source of the nucleic acid molecules. The term "nucleic acid library" as used herein refers to a collection of DNA molecules derived from and representing all or part of the genetic material of an organism, tissue, or cell. Examples of nucleic acid libraries are genomic libraries, which are derived from restriction fragments of a genome, and cDNA libraries, which are derived from the mRNA of an organism, tissue, or cell. In preferred embodiments, nucleic acid libraries can be developmentally specific, *i.e.*, derived from a specific developmental stage, cell lineage specific, *i.e.*, derived from a specific cell lineage, and/or tissue specific, *i.e.*, derived from a specific tissue.

In yet other embodiments, the invention relates to methods for identifying a developmentally competent nuclear donor cell line, using the ESTs and gene expression databases of the invention.

These methods can comprise: performing one or more nuclear transfer procedures using cells(s) separated from a cell line to provide one or more nuclear transfer embryos; culturing each of the nuclear transfer embryos to at least two cells; separating at least one cell from each of the cultured embryos; determining the developmental competence of each of the separated embryonic cells by comparing one or more nucleic acid molecules from each embryonic cell to a gene expression database; and identifying those embryos resulting from nuclear transfer of a developmentally competent nuclear donor cell.

The term "separating" as used herein refers to isolating one or more cells from a cell mass or cell culture. Cells can be separated by mechanical and chemical means well known to the skilled artisan. Cells can also be separated, for example, by biopsy or needle aspiration of a cell mass or cell culture. In this context, a "cell mass" can refer to an embryo, a fetus, or an animal.

The term "determining the developmental competence" with respect to embryos refers to identifying if an embryo is capable of developing into a live born animal. In certain embodiments, developmental competence is determined by implanting an embryo in a maternal host and allowing the embryo to develop until it either terminates or results in a live born animal. In certain other embodiments, developmental competence is determined by comparing the nucleic acid sequences present and/or expressed in one or more cells of an embryo to one or more nucleic acids that identify a cell as "developmentally competent" or "developmentally incompetent." Preferably, this determination is made using the ESTs and gene expression databases described herein. Cells obtained from an embryo can be used directly to determine the developmental competence of the source embryo, or the cells can be cultured prior to their use.

In preferred embodiments, an embryo is determined to be developmentally competent based on the presence in one or more cells obtained from the embryo of one or more nucleic acid sequences that are known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence. As discussed herein, expression of nucleic acid sequences in a cell may be indirectly detected by detecting molecules produced from the nucleic acid sequences, such as proteins or peptides.

In other preferred embodiments, an embryo is determined to be developmentally incompetent based on the presence in a cell obtained from the embryo of one or more nucleic acid sequences that are that are present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent.

In yet other preferred embodiments, an embryo is determined to be developmentally competent based the presence in a cell obtained from the embryo of one or more nucleic acid sequences that are known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that are present

and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence; and the absence of one or more nucleic acid sequences that are that are present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent.

In additional preferred embodiments, an embryo is determined to be developmentally incompetent based the presence in a cell obtained from the embryo of one or more nucleic acid sequences that are that are present and/or expressed in a cell line that has been tested for, but has failed to demonstrate developmental competence, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be developmentally competent; and the absence of one or more nucleic acid sequences that are known to be present and/or expressed in a cell line that has been demonstrated to be developmentally competent, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate developmental competence.

In additional embodiments, the invention relates to methods for producing one or more embryos, fetuses, and/or animals by nuclear transfer procedures using a developmentally competent nuclear donor cell.

Methods for preparing embryos according to the invention preferably comprise: performing one or more nuclear transfer procedures using cells(s) separated from a cell line to provide one or more nuclear transfer embryos; culturing each of the nuclear transfer embryos to at least two cells; separating at least one cell from each of the cultured embryos; determining the developmental competence of each of the separated embryonic cells by comparing one or more nucleic acid molecules from each embryonic cell to a gene expression database; and identifying those embryos resulting from nuclear transfer of a developmentally competent nuclear donor cell. Preferred methods for preparing fetuses and/or animals further comprise implanting the identified embryos into one or more recipient females for development into one or more fetuses and/or animals.

The term "culturing" as used herein in reference to embryos refers to laboratory procedures that involve placing an embryo in a culture medium. An embryo can be placed in a culture medium for an appropriate amount of time to allow stasis of an embryo, or to allow the embryo to grow in the medium. Culture media suitable for culturing embryos are well-known to those skilled in the art. *See, e.g.,* Nagashima *et al.*, 1997, *Mol. Reprod. Dev. 48*: 339-343; Petters & Wells, 1993, *J. Reprod. Fert. (Suppl) 48*: 61-73; Reed *et al.*, 1992, *Theriogenology 37*: 95-109; Dobrinsky *et al.*, 1996, *Biol. Reprod. 55*: 1069-1074; U.S. Patent No. 5,213,979, First *et al.*, "In Vitro Culture of Bovine Embryos," May 25, 1993; U.S. Patent No. 5,096,822, Rosenkrans, Jr. *et al.*, "Bovine Embryo Medium," March 17, 1992, each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings. Alternatively, an embryo may be "cultured *in vivo,*" for example by placing the embryo into the ligated oviduct of a recipient female, for an appropriate amount of time to allow stasis of an embryo, or to allow the embryo to grow. Techniques of culturing an embryo *in vivo* are well known to those skilled in the art.

The term "suitable medium" as used herein refers to any medium that allows cell proliferation or allows stasis of an embryo. If a medium allows cell proliferation, a suitable medium need not promote maximum proliferation, only measurable cell proliferation. A suitable medium for embryo development can be an embryo culture medium described herein by example. Embryos of the invention can be cultured in media with or without feeder cells. In preferred embodiments, the feeder cells can be cumulus cells.

The terms "maternal recipient" and "recipient female" as used herein refers to a female animal which is implanted with an embryo for development of the embryo. A maternal recipient may be either homospecific or xenospecific to the implanted embryo. For example it has been shown in the art that bovine embryos can develop in the oviducts of sheep. Stice & Keefer, 1993, "Multiple generational bovine embryo cloning," *Biology of Reproduction 48*: 715-719. Implanting techniques are well known to a person of ordinary skill in the art. *See, e.g.*, Polge & Day, 1982, "Embryo transplantation and preservation," *Control of Pig Reproduction*, DJA Cole and GR Foxcroft, eds., London,

UK, Butterworths, pp. 227-291; Gordon, 1997, "Embryo transfer and associated techniques in pigs," *Controlled reproduction in pigs* (Gordon, ed), CAB International, Wallingford UK, pp 164-182; and Kojima, 1998, "Embryo transfer," *Manual of pig embryo transfer procedures,* National Livestock Breeding Center, Japanese Society for Development of Swine Technology, pp 76-79, each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

In preferred embodiments (1) an embryo, fetus, or animal of the present invention is a mammalian embryo, fetus, or animal; (2) a mammal is selected from the group consisting of canids, felids, murids, leporids, ursids, mustelids, and human and non-human primates; (3) a mammal is an ungulate; and (4) an ungulate is selected from the group consisting of suids, ovids, equids, bovids, caprids, and cervids.

In particularly preferred embodiments, embryos, fetuses and/or animals of the invention are transgenic embryos, fetuses and/or animals. The term "transgenic" as used herein in reference to embryos, fetuses and animals refers to an embryo, fetus or animal comprising one or more cells whose genomes has been altered using recombinant DNA techniques. In preferred embodiments, a transgenic embryo, fetus, or animal comprises one or more transgenic cells. While germ line transmission is not a requirement of transgenic embryos, fetuses, or animals as that term is used herein, in particularly preferred embodiments a transgenic embryo, fetus, or animal can pass its transgenic characteristic(s) through the germ line. In certain embodiments, a transgenic embryo, fetus or animal expresses one or more exogenous genes as exogenous RNA and protein molecules. Most preferably, a transgenic embryo, fetus or animal results from a nuclear transfer procedure using a transgenic nuclear donor cell.

Additional embodiments relate to methods for assessing the effect of one or more changes to a nuclear transfer protocol by comparing the developmental competence of nuclear transfer embryos resulting from the changed protocol to the developmental competence of nuclear transfer embryos resulting from a baseline protocol, using the ESTs and expression databases of the invention

Preferably, changes in a nuclear transfer protocol are assessed by: performing one or more nuclear transfer procedures according to a first nuclear transfer protocol to produce one or more first protocol nuclear transfer embryos; performing one or more nuclear transfer procedures according to a second nuclear transfer protocol comprising one or more changes to said first nuclear transfer protocol, to produce one or more second protocol nuclear transfer embryos; determining the developmental competence of each of the first protocol and second protocol nuclear transfer embryos; and assessing the effect of the changes to the protocol by comparing the developmental competence of the first protocol nuclear transfer embryos to the developmental competence of the second protocol nuclear transfer embryos.

The term "assessing the effect of one or more changes in a nuclear transfer protocol" as used herein refers to the process of determining whether changing one or more variables in a nuclear transfer protocol alters the developmental competence of nuclear transfer embryos produced by the protocol. The skilled artisan will understand that the number of variables which may be changed are myriad, and can include changing the donor cell medium composition, the activation parameters, the fusion parameters, the embryo culture parameters, *etc.* By comparing the percentage of developmentally competent embryos produced by a baseline protocol to the percentage of developmentally competent embryos produced by the changed protocol, the effect of the changes can be determined. In preferred embodiments, the effect of the changes to the protocol is to increase the percentage of developmentally competent embryos produced. In certain embodiments, the effect of the changes to the protocol is to decrease the percentage of developmentally competent embryos produced.

The term "comparing the developmental competence" as used herein in reference to embryos refers to determining the percentage of developmentally competent embryos in two different group of embryos, and comparing the relative percentages in the two groups. The term "determining the developmental competence" of embryos is defined herein. In preferred embodiments, a group of embryos for comparison purposed comprise at least 2 embryos, at least 3 embryos, at least 4 embryos, at least 5 embryos, at least 6 embryos, at least 7 embryos, at least 8 embryos, at least 9 embryos, at least 10 embryos,

at least 15 embryos, at least 20 embryos, at least 25 embryos, at least 30 embryos, at least 40 embryos, at least 50 embryos, at least 60 embryos, at least 70 embryos, at least 100 embryos, at least 200 embryos, at least 300 embryos, at least 400 embryos, and at least 500 embryos.

In further embodiments, the invention relates to nucleic acid arrays comprising the ESTs and gene expression libraries of the invention that can be used in methods, such as those described herein, to identify cells, embryos, or fetuses as being developmentally competent or developmentally incompetent.

The term "nucleic acid array" as used herein refers to one or more nucleic acid molecules affixed to a solid matrix. In certain embodiments, nucleic acid arrays can be used as solid supports for hybridization assays. Suitable solid matrices for attaching nucleic acids, and methods of attachment are well known in the art. *See, e.g.*, U.S. Patent No. 6,004,755, issued on December 21, 1999 to B. Wang, entitled "Quantitative Microarray Hybridization Assays;" U.S. Patent No. 5,861,242, issued on January 19, 1999 to Chee *et al.*, entitled "Array of Nucleic Acid Probes on Biological Chips for Diagnosis of HIV and Methods of Using the Same;" U.S. Patent No. 5,830,645, issued on November 3, 1998 to Pinkel *et al.*, entitled "Comparative Fluorescence Hybridization to Nucleic Acid Arrays;" U.S. Patent No. 5,667,976, issued September 16, 1997 to Van Ness *et al.*, entitled "Solid Supports for Nucleic Acid Hybridization Assays;" and U.S. Patent No. 5,215,882, issued on June 1, 1993 to Bahl *et al.*, entitled "Method of Immobilizing Nucleic Acid on a Solid Surface for Use in Nucleic Acid Hybridization Assays," each of which is incorporated in its entirety, including all tables, figures, and claims. In preferred embodiments, a solid phase can be papers, nitrocellulose membranes, nylon membranes, glass, magnetic materials, magnetic beads, polymeric beads, or silicon surfaces. In other preferred embodiments a solid phase can be a solid or semisolid polymer such as polyacrylamide gels and agarose gels.

Preferably, a nucleic acid array comprises at least one nucleic acid molecule, the expression of which (or its complementary sequence) identifies a cell as being developmentally competent or developmentally incompetent. More preferably, a nucleic

acid array comprises from 2 to 10,000 or more nucleic acid molecules, the expression of which (or their complementary sequences) identifies a cell as being developmentally competent or developmentally incompetent. In particularly preferred embodiments, a nucleic acid array comprises at least one nucleic acid molecule, the expression of which (or its complementary sequence) identifies a cell as being developmentally competent, and at least one nucleic acid molecule, the expression of which (or its complementary sequence) identifies a cell as being developmentally incompetent.

In particularly preferred embodiments, a cell, embryo or fetus is identified as developmentally competent based on the presence of complementary sequences to at least about 75% of the ESTs comprised in such a nucleic acid array; at least about 90% of the ESTs comprised in such a nucleic acid array; at least about 95% of the ESTs comprised in such a nucleic acid array; and about 100% of the ESTs comprised in such a nucleic acid array.

In a second aspect, the invention concerns identifying and using one or more expressed sequence tags, the expression of which can be used to identify a cell, most preferably a stem cell, as being capable of committing to a specific cell lineage.

In certain embodiments, cells, and most preferably stem cells, can be identified as being capable of committing to a specific cell lineage based on the expression of an expressed sequence tag (or its complementary sequence) known to be present and/or expressed in a cell line that has been demonstrated to be capable of committing to that cell lineage, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate such a capability. Similarly, cells can be identified as being incapable of committing to a specific cell lineage based on the expression of an expressed sequence tag (or its complementary sequence) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate the capability of committing to the cell lineage, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be capable of committing to that cell lineage.

The term "stem cell" as used herein refers to one or more cells capable of differentiating into one or more different cell lineages. For example, hematopoetic stem cells can differentiate into one or more different blood cell types such as erythrocytes, platelets, macrophages, lymphocytes, *etc.* Such cells are pluripotent. Alternatively, some stem cells differentiate into a single cell lineage. For example, epidermal stem cells can differentiate into cornified epidermal cells. Such cells are unipotent. In particularly preferred embodiments, a stem cell is an embryonic stem cell.

The term "embryonic stem cell" as used herein refers to pluripotent cells isolated from an embryo that are maintained in *in vitro* cell culture. Embryonic stem cells may be, but need not be, totipotent. Embryonic stem cells may be cultured with or without feeder cells. Embryonic stem cells can be established from embryonic cells isolated from embryos at any stage of development, including blastocyst stage embryos and pre-blastocyst stage embryos. Embryonic stem cells may have a rounded cell morphology and may grow in rounded cell clumps on feeder layers. Embryonic stem cells are well known to a person of ordinary skill in the art. *See, e.g.*, WO 97/37009, entitled "Cultured Inner Cell Mass Cell-Lines Derived from Ungulate Embryos," Stice and Golueke, published October 9, 1997, and Yang & Anderson, 1992, *Theriogenology 38*: 315-335, each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings. *See, also, e.g.*, Piedrahita *et al.,* 1998, *Biol. Reprod. 58*: 1321-1329; Wianny *et al.,* 1997, *Biol. Reprod. 57*: 756-764; Moore & Piedrahita, 1997, *In Vitro Cell Biol. Anim. 33*: 62-71; Moore, & Piedrahita, 1996, *Mol. Reprod. Dev. 45*: 139-144; Wheeler, 1994, *Reprod. Fert. Dev. 6*: 563-568; Hochereau-de Reviers & Perreau, *Reprod. Nutr. Dev. 33*: 475-493; Strojek *et al.,* 1990, *Theriogenology 33*: 901-903; Piedrahita *et al.,* 1990, *Theriogenology 34*: 879-901; and Evans *et al.,* 1990, *Theriogenology 33*: 125-129, each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

In preferred embodiments (1) a stem cell or a stem cell line of the present invention is a mammalian stem cell or stem cell line; (2) a mammalian stem cell or stem cell line is selected from the group consisting of canid stem cells or stem cell lines, felid stem cells or stem cell lines, murid stem cells or stem cell lines, leporid stem cells or stem

cell lines, ursid stem cells or stem cell lines, mustelid stem cells or stem cell lines, and human and non-human primate stem cells or stem cell lines; (3) a mammalian stem cells or stem cell lines is an ungulate stem cells or stem cell lines; and (4) an ungulate stem cells or stem cell lines is selected from the group consisting of suid stem cells or stem cell lines, ovid stem cells or stem cell lines, equid stem cells or stem cell lines, bovid stem cells or stem cell lines, caprid stem cells or stem cell lines, and cervid stem cells or stem cell lines.

The term "differentiated" as used herein refers to a cell that has developed from an unspecialized phenotype to a specialized phenotype.

The term "undifferentiated cell" as used herein refers to a precursor cell that has an unspecialized phenotype and is capable of differentiating. An example of an undifferentiated cell is a stem cell.

The term "committing to a specific cell lineage" as used herein refers to the ability of a cell to differentiate into a specific cell type. For example, a hematopoetic stem cell may be capable of committing to an erythrocyte, platelet, macrophage, lymphocyte, *etc.*, lineage, while an embryonic stem cell may be capable of committing to a wider variety of cell lineages, such as a muscular, neuronal, hematopoetic, osteal, germinal, *etc.*, cell lineage. A cell, and in particular a stem cell, may be capable of committing to certain cell lineages, yet incapable of committing to others. A cell may only commit to a specific cell lineage when exposed to a proper differentiation-inducing stimulus.

As discussed above, the term "identifies" or "identifying" as used herein with respect to cells refers to the ability to distinguish between cells having two distinct characteristics. In preferred embodiments, a cell or cell line that is capable of committing to a specific cell lineage can be distinguished from that is not capable of committing to that lineage. In certain preferred embodiments, an EST or ESTs identify a cell or cell line as "capable of committing to a specific cell lineage" if the EST sequences are present and/or expressed in stem cells known to be capable of committing to that lineage, but are present and/or expressed at a reduced or nondetectable level in stem cells that have been tested for, but have failed to demonstrate the ability to commit to that lineage. Similarly,

an EST or ESTs identify a cell or cell line as "incapable of committing to a specific cell lineage" if the sequences are present and/or expressed in embryos in stem cells that have been tested for, but have failed to demonstrate the ability to commit to that lineage, but are present and/or expressed at a reduced or nondetectable level in known to be capable of committing to that lineage.

In additional embodiments, expressed sequence tags can be grouped in numbers of 2 or more, and up to numbers of 10,000 or more, to provide a gene expression database. The expression of one or more expressed sequence tags in the database can be used to identify cells, most preferably stem cells, capable of committing to a specific cell lineage.

Preferably, a gene expression database comprises two or more expressed sequence tags (or their complementary sequences) known to be present and/or expressed in a cell line that has been demonstrated to be capable of committing to a specific cell lineage, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate such a capability. Cells can be identified as capable of committing to a specific cell lineage based on the presence of at least one of the ESTs in such a gene expression database. In particularly preferred embodiments, cells can be identified as capable of committing to a specific cell lineage based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Likewise, a gene expression database preferably comprises two or more expressed sequence tags (or their complementary sequences) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate the capability of committing to a specific cell lineage, but that are present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be capable of committing to that cell lineage. Cells can be identified as incapable of committing to a specific cell lineage based on the presence of at least one of the ESTs in such a gene expression

database. In particularly preferred embodiments, cells can be identified as incapable of committing to a specific cell lineage based on the presence of at least about 75% of the ESTs in such a gene expression database; at least about 90% of the ESTs in such a gene expression database; at least about 95% of the ESTs in such a gene expression database; and about 100% of the ESTs in such a gene expression database.

Most preferably, a gene expression database comprises at least one EST (or its complementary sequence) known to be present and/or expressed in a cell line that has been demonstrated to be capable of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been tested for, but has failed to demonstrate such a capability; and at least one EST (or its complementary sequence) known to be present and/or expressed in a cell line that has been tested for, but has failed to demonstrate the capability of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in a cell line that has been demonstrated to be capable of committing to that cell lineage. In such embodiments, cells can be identified as capable of committing to a specific cell lineage based on the presence on the presence of at least one of the former ESTs, and the absence of the latter ESTs. Likewise, cells can be identified as incapable of committing to a specific cell lineage based on the presence of at least one of the latter ESTs, and the absence of the former ESTs.

In further embodiments, the invention concerns methods for identifying one or more expressed sequence tags, the expression of which can be used to identify cells, most preferably stem cells, capable of committing to a specific cell lineage.

In preferred embodiments, one or more ESTs are identified by comparing one or more first nucleic acid molecules obtained from one or more cell lines that have been demonstrated to be capable of committing to a specific cell lineage to one or more second nucleic acid molecules obtained from one or more cell lines that have been tested for, but have failed to demonstrate such a capability. ESTs that signal the capability to commit to a specific cell lineage are identified as one or more nucleic acid molecules that are present in the population of first nucleic acid molecules, but that are not present at a

detectable level in the population of second nucleic acid molecules. Likewise, one or more nucleic acid molecules that are present in the population of second nucleic acid molecules, but that are not present at a detectable level in the population of first nucleic acid molecules are identified as ESTs that signal the incapability to commit to a specific cell lineage.

In particularly preferred embodiments, an EST that signals the capability to commit to a specific cell lineage is a nucleic acid molecule (1) present and/or expressed in at least about 75% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 75% of cell lines that have been tested for, but have failed to demonstrate such a capability; (2) present and/or expressed in at least about 90% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 90% of cell lines that have been tested for, but have failed to demonstrate such a capability; (3) present and/or expressed in at least about 95% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 95% of cell lines that have been tested for, but have failed to demonstrate such a capability; and (4) present and/or expressed in at least about 100% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 100% of cell lines that have been tested for, but have failed to demonstrate such a capability.

In other particularly preferred embodiments, an EST that signals the incapability to commit to a specific cell lineage is a nucleic acid molecule (1) present and/or expressed in at least about 75% of cell lines that have been tested for, but have failed to demonstrate the ability to commit to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 75% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage; (2) present and/or expressed in at least about 90% of cell lines that have been tested for, but have failed to demonstrate the ability to commit to a specific cell lineage, but that is present

and/or expressed at a reduced or nondetectable level in at least about 90% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage; (3) present and/or expressed in at least about 95% of cell lines that have been tested for, but have failed to demonstrate the ability to commit to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 95% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage; and (4) present and/or expressed in at least about 100% of cell lines that have been tested for, but have failed to demonstrate the ability to commit to a specific cell lineage, but that is present and/or expressed at a reduced or nondetectable level in at least about 100% of cell lines that have been demonstrated to be capable of committing to a specific cell lineage.

In another aspect, the invention concerns methods that identify one or more molecules that affect the developmental competence of cells, cell lines, embryos, fetuses, and/or animals.

In certain embodiments, molecules can be identified that induce developmental competence in an otherwise developmentally incompetent cell line. Similarly, molecules can be identified that induce developmental incompetence in an otherwise developmentally competent cell line. Such molecules can be used to increase the availability of developmentally competent cells for use as nuclear donor cells in nuclear transfer procedures, for the treatment of certain diseases, or for preventing full term pregnancies.

In preferred embodiments, molecules that induce developmental competence in an otherwise developmentally incompetent cell line can be identified by: contacting a developmentally incompetent cell line with one or more molecules to provide a treated cell line; separating one or more cells from the treated cell line to provide one or more separated cells; performing one or more nuclear transfer procedures using one or more separated cells to provide one or more nuclear transfer embryos; and determining the developmental competence of each of the nuclear transfer embryos. In particularly preferred embodiments, developmental competence is determined by comparing a

plurality of nucleic acid molecules obtained from each of the embryos to a gene expression database of the instant invention.

Likewise, molecules that induce developmental incompetence in an otherwise developmentally competent cell line can be identified by: contacting a developmentally competent cell line with one or more molecules to provide a treated cell line; separating one or more cells from the treated cell line to provide one or more separated cells; performing one or more nuclear transfer procedures using one or more separated cells to provide one or more nuclear transfer embryos; and determining the developmental competence of each of the nuclear transfer embryos. In particularly preferred embodiments, developmental competence is determined by comparing a plurality of nucleic acid molecules obtained from each of the embryos to a gene expression database of the instant invention.

The term "contacting" as used herein with respect to cells refers to bringing one or more cells together with one or more molecules, whether in an *in vitro* system (*e.g.*, in a test tube or an *ex vivo* system) or an *in vivo* system. One or more cells may be removed from an organism for contacting with one or more molecules, and then the cells can be returned to the same or a different animal.

In further embodiments, one or more molecules identified as inducing or inhibiting developmental competence can be used to induce or inhibit developmental competence in cells, cell lines, embryos, fetuses, or animals, by administering one or more molecules so identified to cells, cell lines, embryos, fetuses, or animals. In particularly preferred embodiments, administering one or more molecules so identified can be used to treat diseases in an animal, embryo, or fetus, or to prevent a full term pregnancy.

The term "administering" as used herein refers to a method of contacting one or more molecules with the one or more cells, cell lines, embryos, fetuses, or animals. In the case of embryos, fetuses, and animals, cells may be contacted with one or more molecules while within an embryo, fetus, or animal; or cells may be removed from the embryo, fetus, or animal, contacted with one or more molecules, and then returned to the

same or a different embryo, fetus, or animal. The compound can be prepared using a carrier such as dimethyl sulfoxide (DMSO) in an aqueous solution or preparation. The compounds may be administered to cells or tissues using a suitable buffered solution. Cells existing outside an organism can be maintained or grown in cell culture dishes. For cells harbored within an organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, ocular, subcutaneous, and rectal applications. For cells outside of the organism, multiple techniques exist in the art to administer the compounds, including (but not limited to) cell microinjection techniques, transformation techniques, and carrier techniques.

In particularly preferred embodiments, one or more molecules can be administered to one or more cultured or non-cultured embryonic cells, embryonic stem cells, inner cell mass cells, fetal cells, embryonic germ cells, somatic cells, adult cells, neurons, glial cells, muscle cells, bone marrow cells, stem cells, hepatocytes, renal cells, muscle cells, cardiac cells, epidermal cells, oocytes, fertilized oocytes, spermatocytes, nuclear transfer embryos, pancreatic cells, lymphocytes, tumor cells, malignant cells, teratoma cells, seminoma cells, carcinoma cells, lymphoma cells, glioblastoma cells, hepatocellular carcinoma cells, and hamartoma cells.

The term "pharmaceutically acceptable composition" refers to a preparation comprising one or more molecules. The composition is acceptable if it does not appreciably cause irritations to the organism administered the composition.

The term "suitable buffered solution" refers to an aqueous preparation of a molecule that comprises a salt that can control the pH of the solution at low concentrations. Because the salt exists at low concentrations, the salt preferably does not alter the function of cells.

In another aspect, the invention concerns methods that identify and use one or more molecules that induce lineage specific development in a cell line, most preferably a stem cell line.

In certain embodiments, molecules so identified can the be used to induce lineage-specific development in one or more cells, preferably stem cells or stem cell lines, that might otherwise be incapable of such development.

In preferred embodiments, molecules that induce lineage specific development in a cell line are identified by: contacting a stem cell line known to be capable of differentiation into a specific cell type with one or more molecules to provide a treated cell line; and determining the capability of the treated cell line to differentiate into a specific cell type. In particularly preferred embodiments, the capability of the treated cell line to differentiate into the cell type of interest is determined by comparing a plurality of nucleic acid molecules obtained from one or more treated cells to a gene expression database of the instant invention.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

BRIEF DESCRIPTION OF THE TABLES

Tables 1A and B illustrates data concerning the developmental competence of 59 different nuclear donor cell lines.

Table 2 illustrates EST sequences screened for differential expression in developmentally competent bovine embryos versus developmentally incompetent bovine embryos.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates comparisons of EG+ and ES- donor cell expression profiles, determined using cDNA microarray, differential display, and direct sequencing methods.

Figure 2 illustrates immunoblot analysis of cultured EG+ and ES- donor cells.

Figure 3 illustrates examples of differential display analyses comparing mRNA expression patterns in individual embryos prepared in vivo, or by nuclear transfer using EG+ and ES- donor cells.

Figure 4A illustrates differential display analysis comapring banding patterns of 5 individual in vivo embryos, 6 individual IVF embryos, 5 individual NT embryos and the donor cell line (DC) used to reconstruct NT embryos. Arrows indicate bands present in all *in vivo* and at least 5 of 6 IVF produced embryos. Figure 4B shows a histogram indicating the percentage of bands shared with *in vivo* embryos.

Figure 5 illustrates identical cDNA arrays probed with mRNA representations of a single NT embryo (A) and a single *in vivo* embryo (B). Spots enclosed by circles represent clones detected at high levels in a single *in vivo* embryo and a single NT embryo reconstructed using a competent donor cell line, but at low levels (or undetected) in single NT embryos reconstructed from incompetent donor cell lines and an unknown cell line.

Figure 6 illustrates a profile of the cDNA clones used for microarray analysis.

Figure 7 illustrates cluster analysis performed on individual embryos prepared by nuclear transfer using developmentally competent and incompetent donor cell lines, and embryos prepared by nuclear transfer using donor cells obtained from a cell line of unknown developmental competence.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to materials and methods for evaluating and affecting the molecular events associated with cellular differentiation and reprogramming, and, in particular, for evaluating and affecting molecular events related to developmental competence and lineage-specific development. The invention provides numerous advantages over methods currently in use. For example, the methods described herein can dramatically increase the number of developmentally competent nuclear donor cells, oocytes, and embryos available. The methods described herein can also dramatically increase the efficiency of nuclear transfer procedures by identifying those nuclear donor cells, oocytes, and embryos most likely to result in successful live births, resulting in an increase in the number of viable embryos, fetuses, and live births, including transgenic embryos, fetuses, and animals. Moreover, the methods described herein can also dramatically increase the efficiency of nuclear transfer procedures by identifying techniques, such as oocyte and embryo maturation, oocyte activation, oocyte

enucleation, timing of implantation, and maternal care most likely to result in successful live births.

As discussed herein, embryos produced by the methods described herein can be used in recloning procedures. Recloned embryos produced by such methods can exhibit enhanced developmental competence compared to embryos produced by a single round of nuclear transfer. In addition, recloning can enhance the efficiency of preparing transgenic embryos, fetuses and/or animals using gene targeting methods. Similarly, fetal cells (*e.g.*, primordial germ cells) can be used as nuclear donor cells in multiple rounds of nuclear transfer for gene targeting methods. Following one or more rounds of nuclear transfer and genetic manipulation, cells obtained from the resulting embryos, fetuses, or animals exhibiting a gene targeting event (such as a knockout or a gene replacement) may be particularly useful as cell-based therapeutics.

Moreover, the materials and methods described herein can increase the efficiency at which cells, and particularly stem cells, can be induced to differentiate into a specific cell lineage. Particularly when coupled with the ability to perform gene targeting with increased efficiency, the instant methods can greatly foster development of cell-based therapeutics.

I. Obtaining and Using Tissue-Specific and Developmentally-Specific Marker Genes and Sequences

The instant invention describes methods to evaluate molecular events associated with cellular reprogramming and differentiation. The tissue-specific and developmentally-specific marker molecules described by the instant invention can be any molecules that are expressed differentially as cells undergo reprogramming to a developmentally competent state, or as cells commit to a specific differentiation pathway. Preferably, such marker molecules are nucleic acid molecules, such as mRNAs, or cDNAs obtained therefrom; however, downstream products of these nucleic acids, such as proteins resulting from translation of mRNAs, or products produced by those proteins, can also be associated with cellular reprogramming and differentiation by techniques well known to the skilled artisan.

A. Expressed Sequence Tags

Methods for identifying and isolating expressed sequence tags are well known to the ordinarily skilled artisan. mRNAs, or cDNAs prepared therefrom, are preferred as a source of expressed sequence tags, as these molecules represent the expressed subset of genomic nucleic acid sequences. Preferably, full length or partial length cDNA clones can be prepared from one or more cells, embryos, fetuses, tissues, or animals by methods such as those described in Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, NY; and Innis, *et al.*,1990, *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA. If necessary, RNA molecules that are present in only low abundance can be amplified by methods well known to those of skill in the art. *See, e.g.*, Innis, *et al.*,1990.

B. Identifying Differentially Expressed Nucleic Acid Molecules

Tissue-specific and developmentally-specific nucleic acid molecules can be identified by comparing the mRNA or cDNA populations obtained from cells in two different differentiation or developmental states. Numerous methods are known to the skilled artisan for identifying commonly expressed and differentially expressed nucleic acid molecules. For example, northern analysis, nucleic acid sequencing, and S1 nuclease protection assays can be used to quantitate relative gene expression levels. Preferably, relative copy numbers of target nucleic acids can be determined as described in U.S. Patent No. 5,830,645, issued to Pinkel *et al.* on November 3, 1998, entitled "Comparative Fluorescence Hybridization to Nucleic Acid Arrays;" gene subtraction methods and differential display methods can identify sequences differeing in or common to two nucleic acid populations as described in U.S. Patent No. 5,436,142, issued to Wigler *et al.* on July 25, 1995, entitled "Methods for Producing Probes Capable of Distingushing Variant Genomic Sequences," Liang and Pardee, 1997, *Meth. Mol. Biol.* **85**: 3-11; and U.S. Patent No. 5,935,788, issued to Burmer *et al.* on August 10, 1999, entitled "Subtractive Hybridization Techniques for Identifying Differentially Expressed and Commonly Expressed Nucleic Acid;" and differential display PCR or RT-PCR can identify sequences differeing in or common to two nucleic acid populations as described

in U.S. Patent 5,773,213, issued to Gullans *et al.* on June 30, 1998, entitled "Method for Conducting Sequential Nucleic Acid Hybridization Techniques." Each of the references cited in this section are hereby incorporated by reference in their entirety, including all figures, tables, and claims.

C. Hybridization Supports

Nucleic acid hybridization techniques, such as those described herein, can be performed by methods that are well known to the ordinarily skilled artisan. *See, e.g.*, Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, NY; and U.S. Patent No. 5,215,882, issued to Bahl *et al.* on June 1, 1993, entitled "Method of Immobilizing Nucleic Acid on a Solid Surface For Use In Nucleic Acid Hybridization Assays." These methods can typically rely on affixing a test nucleic acid on a solid surface, such as cellulose or nylon papers or membranes or glass slides, which acts as a support for the hybridization assay. Numerous hybridization supports are known in the art. Particularly preferred hybridization supports are polymer beads and dipsticks, such as those described in U.S. Patent No. 5,667,976, issued to Van Ness *et al.* on September 16, 1997, entitled "Solid Supports for Nucleic Acid Hybridization Assays;" and nucleic acid arrays, macroarrays, and microarrays, such as those described in U.S. Patent No. 5,861,242, issued to Chee *et al.* on January 19, 1999, entitled "Array of Nucleic Acid Probes on Biolological Chips For Diagnosis of HIV and Methods of Using Same;" and U.S. Patent No. 6,004,755, issued to Wang on December 21, 1999, entitled "Quantitative Microarray Hybridization Assays." Each of the references cited in this section are hereby incorporated by reference in their entirety, including all figures, tables, and claims.

D. Correlating Differentially Expressed Nucleic Acid Molecules to Cellular Reprogramming

The expression patterns of tissue-specific and developmentally-specific marker molecules can be analyzed to determine their correlation to characteristics such as developmental competence or incompetence, or to the ability to differentiate along a given lineage, using techniques well known to the skilled artisan. For example, Pearson

correlation, as described in Golub *et al.*, 1999, *Science* **286**: 531-7; hierarchical clustering as described in (Iyer et. al., '99) ; and Euclidian distance analysis as described in Golub *et al.*, 1999, *Science* **286**: 531-7 can be used to predict which marker molecules are most closely related to a given characteristic.

Preferably, neighbor analysis as described in Golub *et al.*, 1999, *Science* **286**: 531-7, can be used to identify an idealized expression pattern that predicts a given characteristic. In this method, differences between classes relative to the standard deviation with each class are considered. Each gene or EST is represented by an expression vector $e_g=(e_{g1}, e_{g2}, e_{g3},\ldots,e_{gs})$, where e_{gi} denotes the log expression level of gene g in the i^{th} sample, for a total of s samples on two classes. The statistic $P(g,c)=[\mu_1(g) - \mu_2(g)]/[\sigma_1(g) + \sigma_2(g)]$, where $\mu_k(g)$ and $\sigma_k(g)$ denote the mean and standard deviation of the log expression levels of gene g across S_k samples in class k relates to the degree of correlation between a gene or EST and a given characteristic. Large values of (P(g,c)) indicate a strong correlation, while low values indicate a weak correlation, while the sign indicates in which class the gene or EST is more strongly expressed.

Finally, the observed correlations are examined by neighbor analysis to determine whether the density of genes correlated with a given characteristic is greater than would be predicted by chance.

E. Identifying Differentially Expressed Protein Markers

Tissue-specific and developmentally-specific nucleic acid molecules can be identified and characterized by various protein biochemistry techniques known to the skilled artisan, including immunoblotting, competitive or noncompetitive immunoassay, and immunoprecipitation, and by various nonimmunological methods such as analytical centrifugation, amino acid analysis, sequencing, 1- and 2-dimensional electrophoresis (including both native and denaturing conditions such as SDS-PAGE), chromatography, peptide mapping, nuclear magnetic resonance, electron crystallography, and X-ray crystallography. *See generally*, Deutscher, ed., 1990, *Methods in Enzymology*, Volume 182, Academic Press, San Diego, CA. Particularly preferred methods, comprised under the general heading of "proteomics," and including 2-dimensional electrophoresis

coupled with mass spectroscopy, particularly MALDI-TOF mass spectroscopy, can provide insights into gene expression beyond the mRNA level, including posttranslational modifications that cannot be predicted based solely on a nucleic acid sequence. *See, e.g.*, VanBogelen *et al.*, 1999, *Electrophoresis* **20**: 2149-59; Hatzimanikatis *et al.*, 1999, *Biotech. Prog.* **15**: 312-8; and Blackstock and Weir, 1999, *Trends Biotech.* **17**: 121-7

II. Nuclear Transfer Procedures

Nuclear transfer procedures, i.e., methods in which a full complement of nuclear DNA is introduced from one cell into a second, enucleated, cell are well known to a person of ordinary skill in the art. *See,* U.S. Patent No. 4,994,384 to Prather *et al.*, entitled "Multiplying Bovine Embryos," issued on February 19, 1991; U.S. Patent No. 5,057,420 to Massey, entitled "Bovine Nuclear Transplantation," issued on October 15, 1991; U.S. Patent No. 5,994,619, issued on November 30, 1999 to Stice *et al.*, entitled "Production of Chimeric Bovine or Porcine Animals Using Cultured Inner Cell Mass Cells; U.K. Patents Nos. GB 2,318,578 GB 2,331,751, issued on January 19, 2000 to Campbell *et al.* and Wilmut *et al.*, respectively, entitled "Quiescent Cell Populations For Nuclear Transfer"; and U.S. Patent No. 6,011,197 to Strelchenko *et al.*, entitled "Method of Cloning Bovines Using Reprogrammed Non-Embryonic Bovine Cells," issued on January 4, 2000, each of which are hereby incorporated by reference in its entirety including all figures, tables and drawings.

A. Nuclear Donors

Nuclear donor material used to establish a mammalian nuclear transfer embryo can be obtained from a variety of cell types, including cultured and non-cultured cells isolated from an embryo arising from the union of two gametes in vitro or in vivo; cultured and non-cultured pluripotent cells, such as embryonic stem cells (ES cells) arising from cultured embryonic cells (*e.g.*, pre-blastocyst cells and inner cell mass cells); cultured and non-cultured cells arising from inner cell mass cells isolated from embryos; cultured and non-cultured pre-blastocyst cells; cultured and non-cultured fetal cells; cultured and non-cultured primordial germ cells; cultured and non-cultured germ cells

(*e.g.*, embryonic germ cells); cultured and non-cultured somatic cells isolated from an animal or fetus; cultured and non-cultured cumulus cells; cultured and non-cultured amniotic cells; cultured and non-cultured fetal fibroblast cells; cultured and non-cultured genital ridge cells; cultured and non-cultured differentiated cells; cultured and non-cultured cells in a synchronous population; cultured and non-cultured cells in an asynchronous population; cultured and non-cultured serum-starved cells; cultured and non-cultured permanent cells; and cultured and non-cultured totipotent cells.

Particularly preferred mammalian nuclear donor cells are canids, felids, murids, leporids, mustelids, ursids, human and non-human primates, ungulates, ovids, suids, equids, bovids, caprids, and cervids. While pluripotent nuclear donor cells can typically give rise to the cloned embryos of the invention, a totipotent nuclear donor cell is generally preferable. For nuclear transfer techniques, a donor cell may be separated from a growing cell mass, isolated from a primary cell culture, and/or isolated from a cell line. An entire cell may be placed in the perivitelline space of a recipient oocyte or may be directly injected into a recipient oocyte by aspirating the nuclear donor into a needle or a Piezo drill, placing the needle/drill tip into a recipient oocyte, releasing the nuclear donor and removing the needle without significantly disrupting the plasma membrane of the oocyte. Also, a nucleus (*e.g.*, a karyoplast) may be isolated from a nuclear donor and placed into the perivitelline space of a recipient oocyte or may be injected directly into a recipient oocyte, for example.

A variety of methods for culturing nuclear donor cells exist in the art. See, *e.g.*, Culture of Animal Cells: a manual of basic techniques (3rd edition), 1994, Freshney (ed.), Wiley-Liss, Inc.; Cells: a laboratory manual (vol. 1), 1998), Spector, Goldman, Leinwand (eds.), Cold Spring Harbor Laboratory Press; and Animal Cells: culture and media, 1994, Darling & Morgan, John Wiley and Sons, Ltd., each of which is incorporated herein by reference in its entirety including all figures, tables, and drawings.

B. Transgenic Nuclear Donor Cells

Materials and methods readily available to a person of ordinary skill in the art can be utilized to convert the nuclear donor cells of the invention (*e.g.*, amniotic cells and

follicular cells) into transgenic cells. Once nuclear DNA is modified in a nuclear donor cell, embryos, fetuses, and animals arising from these cells can also comprise the modified nuclear DNA. Hence, materials and methods readily available to a person of ordinary skill in the art can be applied to nuclear donor cells to produce transgenic cloned and chimeric animals. See, *e.g.*, EPO 264 166, entitled "Transgenic Animals Secreting Desired Proteins Into Milk"; WO 94/19935, entitled "Isolation of Components of Interest From Milk"; WO 93/22432, entitled "Method for Identifying Transgenic Pre-implantation Embryos"; WO 95/17085, entitled "Transgenic Production of Antibodies in Milk;" Hammer *et al.*, 1985, Nature 315: 680-685; Miller *et al.*, 1986, J. Endocrinology 120: 481-488; Williams *et al.*, 1992, J. Ani. Sci. 70: 2207-2111; Piedrahita *et al.*, 1998, Biol. Reprod. 58: 1321-1329; Piedrahita *et al.*, 1997, J. Reprod. Fert. (suppl.) 52: 245-254; and Nottle *et al*, 1997, J. Reprod. Fert. (suppl.) 52: 245-254, each of which is incorporated herein by reference in its entirety including all figures, drawings and tables.

Methods for generating transgenic cells typically include (1) assembling a suitable DNA construct useful for inserting a specific DNA sequence into nuclear DNA of a cell; (2) transfecting the DNA sequence into cells; (3) allowing random insertion and/or homologous recombination to occur. A modification resulting from such a process may include insertion of a suitable DNA construct(s) into a target genome; deletion of DNA from a target genome; and/or mutation of a target genome.

DNA constructs can comprise a gene of interest as well as a variety of elements including regulatory promoters, insulators, enhancers, and repressors as well as elements for ribosomal binding to RNA transcribed from a DNA construct. DNA constructs can also encode ribozymes and anti-sense DNA and/or RNA. Moreover, DNA constructs can comprise a selection element, such as a gene for drug selection of transformants. These examples are well known to a person of ordinary skill in the art and are not meant to be limiting.

Due to effective recombinant DNA techniques available in conjunction with DNA sequences for regulatory elements and genes readily available in data bases and the commercial sector, a person of ordinary skill in the art can readily generate a DNA

construct appropriate for establishing transgenic cells using materials and methods described herein. For example, transfection techniques are well known to a person of ordinary skill in the art and materials and methods for carrying out transfection of DNA constructs into cells are commercially available. For example, materials that can be used to transfect cells with DNA constructs are lipophillic compounds, such as Lipofectin™, Superfect™, LipoTAXI™, and CLONfectin™. Particular lipophillic compounds can be induced to form liposomes for mediating transfection of the DNA construct into the cells. In addition, cationic based transfection agents that are known in the art can be utilized to transfect cells with nucleic acid molecules (*e.g.*, calcium phosphate precipitation, DEAE-dextran, polybrene, polyamine). Other techniques are known in the art that use protein-based or amphipathic polyamines as transfection reagents. Also, electroporation techniques known in the art can be utilized to translocate nucleic acid molecules into cells. Furthermore, particle bombardment techniques known in the art can be utilized to introduce exogenous DNA into cells. Target sequences from a DNA construct can be inserted into specific regions of nuclear DNA by rational design of a DNA construct. Such design techniques and methods are well known to a person of ordinary skill in the art. See, U.S. Patent 5,633,067, "Method of Producing a Transgenic Bovine or Transgenic Bovine Embryo," DeBoer *et al.*, issued May 27, 1997; U.S. Patent 5,612,205, "Homologous Recombination in Mammalian Cells," Kay *et al.*, issued March 18, 1997; and PCT publication WO 93/22432, "Method for Identifying Transgenic Pre-Implantation Embryos," each of which is incorporated herein by reference in its entirety, including all figures, drawings, and tables. Once a desired DNA sequence is inserted into the nuclear DNA of a cell, the location of an insertion region as well as the frequency with which the desired DNA sequence has inserted into the nuclear genome can be identified by methods well known to those skilled in the art.

Once a transgene or transgenes are inserted into nuclear DNA of a nuclear donor cell, that cell can be used for cloning a transgenic animal. A description of embodiments related to transgenic animals are described in further detail hereafter.

i. Diseases and Parasites

Desired DNA sequences can be inserted into nuclear DNA of a cell to enhance the resistance of a cloned transgenic animal to particular parasites, diseases, and infectious agents. Examples of parasites include worms, flies, ticks, and fleas. Examples of infectious agents include bacteria, fungi, and viruses. Examples of diseases include Atrophic rhinitis, Cholera, Leptospirosis, Pseudorabies, Pasturellosis, and Brucellosis. These examples are not limiting and the invention relates to any disease or parasite or infectious agent known in the art. See, *e.g.*, Hagan & Bruners Infectious Diesases of Domestic Animals (7th edition), Gillespie & Timoney, copyright 1981, Cornell University Press, Ithaca NY.

A transgene can confer resistance to a particular parasite or disease by completely abrogating or partially alleviating symptoms of the disease or parasitic condition, or by producing a protein which controls the parasite or disease.

ii. Elements of DNA Constructs and Production of DNA Constructs

A wide variety of transcriptional and translational regulatory sequences may be inserted into nuclear DNA of a nuclear donor cell. Transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, whereas the regulatory signals can be associated with a particular gene sequence having a potential for high levels of expression. Additionally, promoters from mammalian expression products, such as actin, casein, alpha-lactalbumin, uroplakin, collagen, myosin, and the like, may be employed. Transcriptional regulatory signals may be selected which allow for repression or activation, so that expression of a gene product can be modulated. Of interest are regulatory signals which can be repressed or initiated by external factors such as chemicals or drugs. These examples are not limiting and the invention relates to any regulatory elements. Other examples of regulatory elements are described herein.

iii. Examples of Preferred Recombinant Products

A variety of proteins and polypeptides can be encoded by a gene harbored within a DNA construct suitable for creating transgenic cells. Those proteins or polypeptides

include hormones, growth factors, enzymes, clotting factors, apolipoproteins, receptors, drugs, pharmaceuticals, bioceuticals, nutraceuticals, oncogenes, tumor antigens, tumor suppressors, cytokines, viral antigens, parasitic antigens, bacterial antigens and chemically synthesized polymers and polymers biosynthesized and/or modified by chemical, cellular and/or enzymatic processes. Specific examples of these compounds include proinsulin, insulin, growth hormone, androgen receptors, insulin-like growth factor I, insulin-like growth factor II, insulin growth factor binding proteins, epidermal growth factor, TGF-α, TGF-β, dermal growth factor, platelet derived growth factor (PDGF), angiogenesis factors (*e.g.*, acidic fibroblast growth factor, basic fibroblast growth factor, and angiogenin), angiogenesis inhibitors (*e.g.*, endostatin and angiostatin), matrix proteins (Type IV collagen, Type VII collagen, laminin), oncogenes (ras, fos, myc, erb, src, sis, jun), E6 or E7 transforming sequence, p53 protein, cytokine receptor, IL-1, IL-6, IL-8, IL-2, α, β, or γ IFN, GMCSF, GCSF, viral capsid protein, and proteins from viral, bacterial and parasitic organisms. Other specific proteins or polypeptides which can be expressed include: phenylalanine hydroxylase, α-1-antitrypsin, cholesterol-7β-hydroxylase, truncated apolipoprotein B, lipoprotein lipase, apolipoprotein E, apolipoprotein A1, LDL receptor, scavenger receptor for oxidized lipoproteins, molecular variants of each, VEGF, and combinations thereof. Other examples are antibodies (monoclonal or polyclonal), antibody fragments, clotting factors, apolipoproteins, drugs, tumor antigens, viral antigens, parasitic antigens, monoclonal antibodies, and bacterial antigens. One skilled in the art readily appreciates that these proteins belong to a wide variety of classes of proteins, and that other proteins within these classes or outside of these classes can also be used. These are only examples and are not meant to be limiting in any way.

It should also be noted that the genetic material which is incorporated into the cells from DNA constructs includes (1) nucleic acid sequences not normally present in target cells; (2) nucleic acid molecules which are normally present in target cells but not expressed at physiological significant levels; (3) nucleic acid sequences normally present in target cells and normally expressed at physiological desired levels; (4) other nucleic

acid sequences which can be modified for expression in target cells; and (5) any combination of the above.

In addition, DNA constructs may become incorporated into nuclear DNA of cells, where incorporated DNA can be transcribed into ribonucleic acid molecules that can cleave other RNA molecules at specific regions. Ribonucleic acid molecules which can cleave RNA molecules are referred to in the art as ribozymes. Ribozymes are themselves RNA molecules. Ribozymes can bind to discrete regions on a RNA molecule, and then specifically cleave a region within that binding region or adjacent to the binding region. Ribozyme techniques can thereby decrease the amount of polypeptide translated from formerly intact message RNA molecules.

Furthermore, DNA constructs can be incorporated into nuclear DNA of cells and when transcribed produce RNA that can bind to both specific RNA or DNA sequences. Nucleic acid sequences can be utilized in anti-sense techniques, where nucleic acids bind to a message (mRNA) in order to block translation. Anti-sense techniques can thereby block or partially block the synthesis of particular polypeptides in cells.

C. Recipient Cells

A recipient cell is a cell into which the nuclear donor is inserted. Preferably, the recipient cell is enucleated, *i.e.*, the recipient cell nucleus chromosomal material is removed or inactivated. A recipient cell is preferably an oocyte with a portion of its ooplasm removed, where the removed ooplasm comprises the oocyte nucleus genetic material. Enucleation techniques are well known to a person of ordinary skill in the art, as described hereafter. Other recipient cells, *e.g.*, a two cell enucleated embryo, are known to the ordinarily skilled artisan. A recipient cell can also be rendered "functionally enucleated," for example by ultraviolet irradiation. *See, e.g.*, Bradshaw *et al.* (1995), Molecular Reproduction and Development 41:503-12.

i. Isolation of Oocytes

Oocytes can be isolated from oviducts and/or ovaries of live animals by oviductal recovery procedures or transvaginal oocyte recovery procedures well known in the art.

Furthermore, oocytes can be isolated from deceased animals. For example, ovaries can be obtained from abattoirs and oocytes can be aspirated from these ovaries. Oocytes can also be isolated from ovaries of a recently sacrificed animal or when an ovary has been frozen and/or thawed. Oocytes may be isolated from ovarian follicles at any stage of development, including primordial follicles, primary follicles, secondary follicles, growing follicles, vesicular follicles, maturing follicles, mature follicles, and graafian follicles. Moreover, oocytes can be isolated from follicles which are obtained from animals, and which are grown and matured in culture. Materials and methods for isolating oocytes from various developmental stages of ovarian follicles are known to those skilled in the art. See, *e.g.*, Laboratory Production of Cattle Embryos, 1994, Ian Gordon, CAB International; Anatomy and Physiology of Farm Animals (5th ed.), 1992, R.D. Frandson and T.L. Spurgeon, Lea & Febiger, each of which is incorporated herein by reference in its entirety including all figures, drawings, and tables.

In preferred embodiments, the recipient oocyte is a mammalian oocyte. Particularly preferred are canid, felid, murid, leporid, mustelid, human and non-human primate, ungulate, ovid, suid, equid, bovid, caprid, and cervid recipient oocytes. A nuclear donor cell and a recipient oocyte may be isolated from an animal of the same species or different species. For example, a porcine cumulus cell can be inserted into a porcine enucleated oocyte. Alternatively, a wild boar cumulus cell can be inserted into a domesticated porcine oocyte. Any nuclear donor/recipient oocyte combinations are envisioned by the invention. Preferably a nuclear donor and recipient oocyte are isolated from the same species. Xenospecific NT techniques can be utilized to produce cloned animals that are endangered or extinct.

Oocytes can be activated by electrical and/or non-electrical means before, during, and/or after a nuclear donor is introduced to recipient oocyte. For example, an oocyte can be placed in a medium containing one or more components suitable for non-electrical activation prior to fusion with a nuclear donor. Also, a cybrid can be placed in a medium containing one or more components suitable for non-electrical activation. Activation processes are discussed in greater detail hereafter.

ii. Oocyte Maturation

Oocytes and cumulus cell/oocyte complexes can be matured in vivo, and more preferably, can be matured in an in vitro environment. The length of time oocytes is matured can vary, depending upon species. In preferred embodiments, oocytes can be matured for (1) greater than about 10 hours; (2) greater than about 20 hours; (3) greater than about 24 hours; (4) greater than about 30 hours; (5) greater than about 40 hours; (6) greater than about 50 hours; (7) greater than about 60 hours (8) greater than about 72 hours; (9) greater than about 80 hours; (10) greater than about 90 hours; and (11) greater than about 100 hours. The term "about" with respect to oocyte maturation refers to plus or minus 5 hours.

A variety of media well known to a person of ordinary skill in the art can be used for maturing oocytes in vitro. See, *e.g.*, (i) Alm & Hinrichs, 1996, J. Reprod. Fert. 107: 215-220 and Alm & Torner, 1994, Theriogenology 42: 345-349 for equine oocytes; (ii) ; Ledda *et al.*, 1997, Journal of Reproduction and Fertility 109:73-78; Byrd *et al.*, 1997, Theriogenology 47: 857-864; Wilmut *et al.*, 1997, Nature 385: 810-813; and LeGal, 1996, Theriogenology 45: 1177-1 for caprine and ovine oocytes; (iii) ; Lorenzo *et al.*, 1996, Journal of Reproduction and Fertility 107:109-117 and Jelinkova *et al.*, 1994, Molecular Reproduction and Development 37:210-215 for leporidine oocytes; (iv) Nickson *et al.*, 1993, J. Reprod. Fert. (Suppl. 47): 231-240; Yamada *et al.*, 1993, J. Reprod. Fert. (Suppl. 47): 227-229; and Mahi &Yanagimachi, 1976, Journal of Experimental Zoology 196; 189-196 for canine oocytes; (v) Fukui *et al.*, 1991, Theriogenology 35: 499-512 and Pollard *et al.*, 1995, Theriogenology 43: 301 for cervidine oocytes; and (vi) Del Campo *et al.*, 1995, Theriogenology 43: 21-30 and Del Campo *et al.*, 1994, Theriogenology 41: 187 for camelid oocytes. One example of such a medium suitable for maturing oocytes in vitro is depicted in an exemplary embodiment described herein. Oocytes can be successfully matured in such a medium within an environment comprising 5% CO_2 at 39°C. Oocytes may be cryopreserved and then thawed before placing the oocytes in maturation medium. Cryopreservation procedures for cells and embryos are well known in the art as discussed herein.

Components of an oocyte maturation medium can include molecules that arrest oocyte maturation. Examples of such components are 6-dimethylaminopurine (DMAP) and isobutylmethylxanthine (IBMX). IBMX has been reported to reversibly arrest oocytes, but the efficiencies of arrest maintenance are quite low. *See, e.g.*, Rose-Hellkant and Bavister, 1996, Mol. Reprod. Develop. 44: 241-249. However, oocytes may be arrested at the germinal vesicle stage with a relatively high efficiency by incubating oocytes at 31°C in an effective concentration of IBMX. Preferably, oocytes are incubated the entire time that oocytes are collected. Concentrations of IBMX suitable for oocyte maturation are 0.01 mM to 20 mM IBMX, preferably 0.05 mM to 10 mM IBMX, and more preferably about 0.1 mM IBMX to about 0.5 mM IBMX, and most preferably 0.1 mM IBMX to 0.5 mM IBMX. The exemplary oocyte maturation procedures are not meant to be limiting and the invention relates to any oocyte maturation procedure known to a person of ordinary skill in the art.

D. Nuclear Transfer

A nuclear donor can be translocated into a nuclear acceptor, preferably an oocyte, most preferably an enucleated oocyte, using a variety of materials and methods that are well known to a person of ordinary skill in the art. In one example, a nuclear donor may be directly injected into a recipient oocyte. This direct injection can be accomplished by gently pulling a nuclear donor into a needle, piercing a recipient oocyte with that needle, releasing the nuclear donor into the oocyte, and removing the needle from the oocyte without significantly disrupting its membrane. Appropriate needles can be fashioned from glass capillary tubes, as defined in the art and specifically by publications incorporated herein by reference.

In another example, at least a portion of plasma membrane from a nuclear donor and recipient oocyte can be fused together by utilizing techniques well known to a person of ordinary skill in the art. *See*, Willadsen, 1986, Nature 320:63-65, hereby incorporated herein by reference in its entirety including all figures, tables, and drawings. Typically, lipid membranes can be fused together by electrical and chemical means, as defined previously and in other publications incorporated herein by reference.

Examples of non-electrical means of cell fusion involve incubating cybrids in solutions comprising polyethylene glycol (PEG), and/or Sendai virus. PEG molecules of a wide range of molecular weight can be utilized for cell fusion.

Processes for fusion that are not explicitly discussed herein can be determined without undue experimentation. For example, modifications to cell fusion techniques can be monitored for their efficiency by viewing the degree of cell fusion under a microscope. The resulting cybrid can then be cloned and identified as totipotent by the methods described below for identifying totipotent cells, which can include tests for selectable markers and/or tests for developing an animal.

E. Activation

Examples of electrical processes for activation are well known in the art. Although electrical pulses are sometimes sufficient for stimulating cell activation, other non-electrical means for activation are useful and are often necessary for proper activation of a cell. Electrical and non-electrical activation may be used separately, or may be used together for activating a cell. Chemical materials and methods useful for non-electrical activation are described below in other preferred embodiments of the invention. When two or more chemical components are introduced to a cell for activating the cell, the components can be added simultaneously or individually in steps.

Examples of components that are useful for non-electrical activation include ethanol; inositol trisphosphate (IP3); divalent ions (*e.g.*, addition of Ca2+ and/or Sr2+); microfilament inhibitors (*e.g.*, cytochalasin B); ionophores for divalent ions (*e.g.*, the Ca2+ ionophore ionomycin); protein kinase inhibitors (*e.g.*, 6-dimethylaminopurine (DMAP)); protein synthesis inhibitors (*e.g.*, cyclohexamide); phorbol esters such as phorbol 12-myristate 13-acetate (PMA); and thapsigargin. It is also known that temperature change and mechanical techniques are also useful for non-electrical activation. The invention includes any activation techniques known in the art. See, *e.g.*, U.S. Patent No. 5,496,720, entitled "Parthenogenic Oocyte Activation," issued on March 5, 1996, Susko-Parrish *et al.*, and Wakayama *et al.*, 1998, Nature 394: 369-374, each of

which is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

Examples of preferred protein kinase inhibitors are protein kinase A, G, and C inhibitors such as 6-dimethylaminopurine (DMAP), staurosporin, 2-aminopurine, sphingosine. Tyrosine kinase inhibitors may also be utilized to activate cells.

Preferred methods for activating cells are depicted in exemplary embodiments described herein. Activation materials and methods that are not explicitly discussed herein can be identified by modifying specified conditions defined in exemplary protocols described hereafter and in U.S. Patent No. 5,496,720.

Activation efficiency and totipotency that result from any modifications of activation procedures can be identified by methods described herein. Methods for identifying totipotent embryos can include one or more tests, such as (a) identifying specific markers for totipotent cells in embryos, and (b) by determining whether embryos are totipotent by allowing them to develop into an animal. Therefore, the invention relates to any modifications to activation procedures described herein even though these modifications may not be explicitly stated herein.

F. Manipulation of Embryos, Fetuses, and Animals Resulting from Nuclear Transfer

An embryo resulting from a NT process can be manipulated in a variety of manners. The invention relates to cloned embryos, fetuses, and animals that arise from at least one NT. Two or more NT procedures may be performed to enhance nuclear transfer efficiency of totipotent embryo, fetus, and animal production and/or placental development. Incorporating two or more NT cycles into methods for cloned embryos, fetuses, and animals can provide further advantages. For example, incorporating multiple NT procedures provides a method for multiplying the number of cloned embryos, fetuses, and animals. Moreover, gene targeting methods require that both copies of a given gene in a diploid cell be targeted in order to knock out or replace the gene. Such methods may require two or more NT procedures in order to efficiently target the gene. The skilled

artisan will understand that the methods required for such manipulations will vary, depending on the species of interest.

When multiple NT procedures are utilized for formation of a cloned embryo, fetus, or animal, oocytes that have been matured for any period of time can be utilized as recipients in the first, second or subsequent NT procedures. For example, if a first NT and then a second NT are performed, the first NT can utilize an oocyte that has been matured for about 53 hours as a recipient and the second NT may utilize an oocyte that has been matured for less than about 53 hours as a recipient. Alternatively, the first NT may utilize an oocyte that has been matured for about 53 hours as a recipient and the second NT may utilize an oocyte that has been matured for greater than about 53 hours as a recipient for a two-cycle NT regime. In addition, both NT cycles may utilize oocytes that have been matured for about 53 hours as recipients, both NT cycles may utilize oocytes that have been matured for less than about 53 hours as recipients, and both NT cycles may utilize oocytes that have been matured for greater than about 53 hours as recipients in a two-cycle NT regime.

For NT techniques that incorporate two or more NT cycles, one or more of the NT cycles may be preceded, followed, and/or carried out simultaneously with an activation step. As defined previously herein, an activation step may be accomplished by electrical and/or non-electrical means as defined herein. An activation step may also be carried out at the same time as a NT cycle (*e.g.*, simultaneously with the NT cycle) and/or an activation step may be carried out prior to a NT cycle. Cloned embryos resulting from a NT cycle can be (1) disaggregated or (2) allowed to develop further.

If embryos are disaggregated, the disaggregated embryonic derived cells can be utilized to establish cultured cells. Any type of embryonic cell can be utilized to establish cultured cells. These cultured cells are sometimes referred to as embryonic stem cells or embryonic stem-like cells in the scientific literature. Embryonic stem cells can be derived from early embryos, morulae, and blastocyst stage embryos. Multiple methods are known to a person of ordinary skill in the art for producing cultured embryonic cells. These

methods are enumerated in specific references previously incorporated by reference herein.

If embryos are allowed to develop into a fetus in utero, or a live-born animal, cells isolated from that fetus or animal can be utilized to establish cultured cells. In preferred embodiments, primordial germ cells, genital ridge cells, and fetal fibroblast cells can be isolated from a fetus. Similarly, a variety of somatic cells or germ cells can be isolated form a live-born animal. Such cultured cells can be established by utilizing culture methods well known to a person of ordinary skill in the art. Such methods are enumerated in publications previously incorporated herein by reference and are discussed herein.

Cloned embryos resulting from NT can also be manipulated by cryopreserving and/or thawing such embryos. See, *e.g.*, Nagashima *et al.*, 1989, Japanese J. Anim. Reprod. 35: 130-134 and Feng *et al.*, 1991, Theriogenology 35: 199, each of which is incorporated herein by reference in its entirety including all tables, figures, and drawings. Other embryo manipulation methods include in vitro culture processes; performing embryo transfer into a maternal recipient; disaggregating blastomeres for NT processes; disaggregating blastomeres or inner cell mass cells for establishing cell lines for use in NT procedures; embryo splitting procedures; embryo aggregating procedures; embryo sexing procedures; and embryo biopsying procedures. Exemplary manipulation procedures are not meant to be limiting and the invention relates to any embryo manipulation procedure known to a person of ordinary skill in the art.

G. Development of Cloned Embryos

i. Culture of Embryos In Vitro

Cloning procedures discussed herein provide an advantage of culturing cells and embryos in vitro prior to implantation into a recipient female. Methods for culturing embryos in vitro are described in detail herein. In addition, exemplary embodiments for media suitable for culturing cloned embryos in vitro are described hereafter. Feeder cell layers may or may not be utilized for culturing cloned embryos in vitro. Feeder cells are described previously and in exemplary embodiments hereafter.

ii. Development of Embryos In Vivo

Cloned embryos can be cultured in an artificial or natural uterine environment after NT procedures. Moreover, cloned embryos can be cultured in vivo prior to, subsequent to, or in the absence of culture of the embryo in vitro. Examples of artificial development environments are being developed and some are known to those skilled in the art. Components of the artificial environment can be modified, for example, by altering the amount of a component or components and by monitoring the growth rate of an embryo.

Methods for implanting embryos into the uterus of an animal are also well known in the art, as discussed previously. Preferably, developmental stage of the embryo(s) is correlated with the estrus cycle of an animal.

Embryos from one species can be placed into a uterine environment in an animal from another species. For example it has been shown in the art that bovine embryos can develop in oviducts of sheep. Stice & Keefer, 1993, "Multiple generational bovine embryo cloning," Biology of Reproduction 48: 715-719. The invention relates to any combination of an embryo in any homospecific or xenospecific uterine environment. A xenospecific in utero development regime can allow for efficient production of cloned animals of an endangered species. For example, a wild boar embryo can develop in the uterus of a domestic porcine sow.

Once an embryo is placed into the uterus of a recipient female, the embryo can develop to term. Alternatively, an embryo can be allowed to develop in the uterus and then can be removed at a chosen time. Surgical methods are well known in the art for removing fetuses from uteri before parturition.

III. Materials and Methods for Oocyte Maturation, Oocyte Enucleation, Cell Activation, In Vitro Embryo Development, and Other Processes

Where descriptions of oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes are described herein in relation to mammals in general, the following references provide additional descriptions of such

process for specific mammals. The following references are provided to aid the reader in understanding the invention and are not admitted to describe or constitute prior art to the present invention. With regard to suids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Grocholová *et al.*, 1997, J. Exp. Zoology 277: 49-56; Schoenbeck *et al.*, 1993, Theriogenology 40: 257-266; Prather *et al.*,1989, Biology of Reproduction 41: 414-418; Prather *et al.*,1991, Molecular Reproduction and Development 28: 405-409; Jolliff & Prather, 1997, Biol. Reprod. 56: 544-548; Mattioli *et al.*,1991, Molecular Reproduction and Development 30: 109-125; Terlouw *et al.*, 1992, Theriogenology 37: 309; Prochazka *et al.*,1992, J. Reprod. Fert. 96: 725-734; Funahashi *et al.*,1993, Molecular Reproduction and Development 36: 361-367; Prather *et al.*, Bio. Rep. Vol. 50 Sup 1: 282; Nussbaum *et al.*, 1995, Molecular Reproduction and Development 41: 70-75; Funahashi *et al.*, 1995, Zygote 3: 273-281; Wang *et al.*, 1997, Biology of Reproduction 56: 1376-1382; Piedrahita *et al.*,1989, Biology of Reproduction 58: 1321-1329; Macháty *et al.*,1997, Biology of Reproduction 57: 85-91; and Macháty *et al.*,1995, Biology of Reproduction 52: 753-758.

With regard to bovids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, U.S. Patents 5,453,357 and 5,670,372, entitled "Pluripotent Embryonic Stem Cells and Methods of Making Same," Hogan; Sims & First, 1993, Theriogenology 39:313; Keefer *et al.*, 1994, Mol. Reprod. Dev. 38:264-268; U.S. Patent No. 4,994,384, "Multiplying Bovine Embryos," Prather *et al.*; U.S. Patent No. 5,057,420, "Bovine Nuclear Transplantation," Massey & Willadsen; Delhaise *et al.*, 1995, Reprod. Fert. Develop. 7:1217-1219; Lavoir 1994, J. Reprod. Dev. 37:413-424; PCT application WO 95/10599 entitled "Embryonic Stem Cell-Like Cells"; Stice *et al.*, 1996, Biol. Reprod. 54: 100-110; Strelchenko, 1996, Theriogenology 45: 130-141; WO 97/37009, entitled "Cultured Inner Cell Mass Cell-Lines Derived from Ungulate Embryos," Stice and Golueke, published October 9, 1997; U.S. Patent No. 5,213,979, entitled "In vitro Culture of Bovine Embryos," First *et al.*, May 25, 1993; U.S. Patent No. 5,096,822, entitled "Bovine Embryo Medium," Rosenkrans, Jr. *et al.*, March 17, 1992; Seidel and Elsden, 1997, Embryo Transfer in Dairy Cattle, W.D. Hoard & Sons, Co., Hoards

Dairyman; Stice & Keefer, 1993, "Multiple generational bovine embryo cloning," Biology of Reproduction 48: 715-719; Wagoner *et al.*, 1996, "Functional enucleation of bovine oocytes: effects of centrifugation and ultraviolet light," Theriogenology 46: 279-284; Pieterse *et al.*, 1988, "Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries," Theriogenology 30: 751-762; Saito *et al.*, 1992, Roux's Arch. Dev. Biol. 201: 134-141; and U.S. Patent No. 5,496,720, entitled "Parthenogenic Oocyte Activation," March 5, 1996, Susko-Parrish *et al.*

With regard to felids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Swanson *et al.*, 1996, Molecular Reprod. Dev. 43: 298-305; Donoghue *et al.*, 1996, J. Reprod. and Fertility 107: 53-58; Goritz *et al.*, 1996, J. Reprod. and Fertility 106: 117-124; Hoffert *et al.*, 1997, Molecular Reprod. Dev. 48: 208-215; Donoghue *et al.*, 1990, Biology of Reprod. 43: 733-744; Wood *et al.*, 1995, J. Reprod. Fertility 104: 315-323; Donoghue *et al.*, 1992, Biology Reprod. 46: 972-980; Johnston *et al.*, 1991, J. Reprod. Fert 92: 377-382; Luvoni *et al.*, 1993, J. Reprod. Fert. Suppl. 47: 203-207; Roth *et al.*, 1997, Biology of Reprod. 57: 165-171; and Jewgenow, 1996, Theriogenology 45: 889-895.

With regard to canids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Nickson *et al.*, 1993, J. Reprod. Fert. (Suppl. 47): 231-240; Yamada *et al.*, 1993, J. Reprod. Fert. (Suppl. 47): 227-229; Mahi &Yanagimachi, 1976, Journal of Experimental Zoology 196; 189-196; Yamada *et al.*, 1992, Biology of Reproduction 46: 853-858; Farstad *et al.*, 1993, Journal of Reproduction and Fertility (Suppl. 47): 219-226; Bolamba *et al.*, 1998, Theriogenology 49: 933-942; Durrant *et al.*, 1998, Theriogenology 49: 917-932; and Hewitt *et al.*, 1998, Theriogenology 49: 1083-1101.

With regard to equids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Alm & Hinrichs, 1996, J. Reprod. Fert. 107: 215-220; Alm & Torner, 1994, Theriogenology 42: 345-349; Hinrichs *et al.*, 1993, Biol. Reprod. 48: 363-

370; Hinrichs *et al.*, 1995, J. Reprod. Fert. 104: 149-156; Hinrichs *et al.*, 1995, Biology of Reproduction Monograph 1: 319-324; and Dell'Aquila *et al.*, 1997, Theriogenology 47: 1139-1156.

With regard to ovids and caprids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Willadsen, 1986, Nature 320: 63-66; Ruffing *et al.*, 1993, Biology of Reproduction 48: 889-904; Smith & Wilmut, 1989, Biology of Reproduction 40: 1027-1035; McLaughlin *et al.*, 1991, Theriogenology 35: 240; Campbell *et al.*, 1995, Theriogenology 43: 181; Cambell *et al.*, 1996, Theriogenology 45: 286; Campbell *et al.*, 1996, Nature 380: 64-66; Wilmut *et al.*, 1997, Nature 385: 810-813; Ledda *et al.*, 1997, Journal of Reproduction and Fertility 109:73-78; Byrd *et al.*, 1997, Theriogenology 47: 857-864; Wilmut *et al.*, 1997, Nature 385: 810-813; LeGal, 1996, Theriogenology 45: 1177-1; Pawshe *et al.*, 1996, Theriogenology 46: 971-982; Gall *et al.*, 1993, Molecular Reproduction and Development 36: 500-506; Walker *et al.*, 1996, Biology of Reproduction 55: 703-708; and Gardner *et al.*, 1994, Biology of Reproduction 50: 390-400.

With regard to murids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. . See, *e.g.*, Downs & Mastropolo, 1997, Mol. Reprod. Dev. 46: 551-566; Kim & Schuetz, 1991, Cell Tissue Res. 265: 105-112; Downs, 1995, Dev. Biol. 167: 502-512; Kito & Bavister; 1997, J. Reprod. Fert. 110: 35-46; Zhang & Rutledge, 1991, Mol. Reprod. Dev. 28: 292-296; Bos-Mickich & Whitingham, 1995, Mol. Reprod. Devel. 42: 254-260; Cuthbertson, 1983, J. Exp. Zool. 226: 311-314; Shaw & Trounson, 1989, Gamete Res. 24: 269-279; Sakkas & Trounson, 1991, Reprod. Fert. Dev. 3: 99-108; Kito & Bavister, 1997, J. Reprod. Fert. 110: 35-46; Bavister, 1995, Human Reprod. Update 1: 91-148; Erbach *et al.*, 1994, Biol. Reprod. 50: 1027-1033; and Ho *et al.*, 1995, Mol. Reprod. Dev. 41: 232-238.

With regard to leporids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and

other processes. See, *e.g.*, Kanka *et al.*, 199, Molecular Reproduction and Development 43: 135-144; Lui *et al.*, 1996, Molecular Reproduction and Development 45: 157-162; Du *et al.*, 1995, Journal of Reproduction and Fertility 104: 219-223; Farrell & Foote, 1995, Journal of Reproduction and Fertility 103: 127-130; Sofikitis *et al.*, 1996, Fertility and Sterility 65: 176-185; Adenot *et al.*, 1997, Molecular Reproduction and Development 46: 325-336; Lorenzo *et al.*, 1996, Journal of Reproduction and Fertility 107:109-117; and Jelinkova *et al.*, 1994, Molecular Reproduction and Development 37:210-215.

With regard to mustelids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Johnston *et al.*, 1994, Journal of Experimental Zoology 269: 53-61; Polejaeva *et al.*, 1997, Journal of Reproduction and Fertility 109: 229-236; and Moreau *et al.*, 1995, Biology of Reproduction 53: 511-518.

With regard to cervids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Berg *et al.*, 1995, Theriogenology 44: 247-254; Berg *et al.*, 1994, Theriogenology 41: 160; Fukui *et al.*, 1991, Theriogenology 35: 499-512; and Pollard *et al.*, 1995, Theriogenology 43: 301.

With regard to camelids, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Del Campo *et al.*, 1995, Theriogenology 43: 21-30; Del Campo *et al.*, 1994, Theriogenology 41: 187; McKinnon *et al.*, 1994, Theriogenology 41: 145-150; Wiepz & Chapman, 1985, Theriogenology 24: 251-257; and Del Campo *et al.*, 1994, Theriogenology 41: 1219-1229.

With regard to non-human primates, researchers have reported materials and methods for oocyte maturation, oocyte enucleation, cell activation, in vitro embryo development, and other processes. See, *e.g.*, Edward, 1965, Nature (Lond) 208: 349-351; Morgan *et al.*, 1991, Biol. Reprod. 45: 89-93; Meng *et al.*, 1997, Biol. Reprod. 57: 454-459; We *et al.*, 1996, Biol. Reprod. 55: 260-270; Bavister *et al.*, 1983, Biol. Reprod. 28: 983-999; Weston *et al.*, 1996, Mol. Reprod. Dev. 44: 88-92; Enders *et al.*, 1989, Biol.

Reprod. 41: 715-727; Wolf *et al.*, 1990, Molec. Reprod. Dev. 27: 261-280; and Zhang *et al.*, 1994, Biol. Reprod. 51: 433-440.

Each reference set forth in this section is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

IV. Cloned Animals

As described previously herein, the invention provides advantages of assessing the genotype and phenotype of an animal before cloning that animal. In preferred embodiments, an animal to be cloned is a mammal. Particularly preferred mammals are canid, felid, murid, leporid, mustelid, ursid, primate, ungulate, ovid, suid, equid, bovid, caprid, and cervid animals. Preferably, a cloned animal has a genome that is substantially similar to that of a nuclear donor cell. More preferably, a cloned animal has a genome that is identical to that of a nuclear donor cell. Differences between a genotype and/or phenotype of the nuclear donor cell and that of the cloned animal can result from natural processes, such as differences in DNA methylation or differences in telomere length for example, and can also result from differences in the intrauterine environment during development. Differences between nuclear donor DNA and that of a cloned animal may also result from the addition of one or more transgenes.

A cloned animal can also be a member of a plurality of animals which share substantially similar, and preferably identical, genomes with a nuclear donor. Differences between a genotype and/or phenotype of each cloned animal and that of other cloned animals descended from a common nuclear donor can result from natural processes, such as differences in DNA methylation or differences in telomere length for example, and can also result from differences in intrauterine environment during development, as described above.

Multiple products can be isolated from a cloned animal. The following discussion of such products is not meant to be limiting and the invention relates to any products which may be isolated or collected from a cloned animal using techniques known to a person of ordinary skill in the art. Products can be any body fluids or organs isolated

from the animal, or any products isolated from fluids or organs. In preferred embodiments, products such as meat may be collected from cloned animals. In preferred embodiments, products may be present in mammary tissue of a female transgenic animal , or present in products of mammary tissue such as breast milk (containing one or more recombinant proteins), which may then be collected and subjected to purification techniques. In another example, semen can be collected from a cloned animal and cryopreserved. Semen can also be separated into sex-specific fractions of sperm. *See*, U.S. Patent Nos. 5,439,362, 5,346,990, and 5,021,244, entitled "Sex-associated Membrane Proteins and Methods for Increasing the Probability that Offspring Will be of a Desired Sex," Spaulding, and issued on August 8, 1995, September 13, 1994, and June 4, 1991, respectively, each of which is incorporated herein by reference in its entirety including all figures, drawings, and tables. Methods of collecting semen are well known to a person of ordinary skill in the art, as discussed previously. In another embodiment, the invention relates to determining a phenotype of an animal, which is a neutered animal, and then cloning this animal such that resulting cloned animals are reproductively functional and can be used to produce semen. Other preferred embodiments of the invention relate to such products as xenograft materials, sperm, embryos, oocytes, any type of cells, and offspring harvested from cloned animals of the invention.

Xenograft materials can relate to any cellular material extracted from one organism and placed into another organism. Medical procedures for extracting the cellular material from one organism and grafting it into another organism are well known to a person of ordinary skill in the art. Examples of preferable xenograft cellular materials can be selected from the group consisting of liver, lung, heart, nerve, brain, gallbladder, kidney, skin, bone, small intestine, large intestine, and pancreas cellular material.

As discussed in a previous section, transgenic animals can be generated from methods of the invention by using transgenic techniques well known to those of ordinary skill in the art. Preferably, cloned transgenic animals are produced from such methods. Cloned transgenic animals can be engineered such that they are resistant or partially resistant to diseases and parasites endemic to such animals. Examples of such diseases and parasites are outlined in a preceding section.

Moreover, cloned transgenic animals can be engineered such that they produce a recombinant product. Examples of recombinant products are outlined in a preceding section. Expression of such products can be directed to particular cells or regions within cloned transgenic animals by selectively engineering a suitable promoter element and other regulatory elements to achieve this end.

For example, human recombinant products can be expressed in urine of cattle by operably linking a uroplakin promoter to the DNA sequence encoding a recombinant product. Alternatively, examples are well known to a person of ordinary skill in the art for selectively expressing human recombinant products in milk of an ungulate animal.

Once a recombinant product or recombinant products have been expressed in a particular tissue or fluid of a cloned transgenic animal, suitable tissue or fluid can be collected using methods well known in the art. Recombinant products can be purified from such fluid or tissue by using standard purification techniques well known to a person of ordinary skill in the art.

V. Method for Treating a Disease or Disorder

The present invention also relates to a method for treating a disease or disorder comprising the step of administering to a patient in need of such a treatment one or more molecules identified as inducing or inhibiting developmental competence or as inducing lineage specific development in a cell line.

Toxicity and therapeutic efficacy of substances, or compounds, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 as determined in cell culture (*e.g.*, the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.* Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and with the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," 1990, 18th ed., Mack Publishing Co., Easton, PA. Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of, for example, tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose,sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

The proper dosage of a compound depends on various factors such as the type of disease being treated, the particular composition being used and the size and physiological condition of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account the IC50 as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

Plasma half-life and biodistribution of the drug and metabolites in the plasma, tumors and major organs can also be determined to facilitate the selection of drugs most appropriate to inhibit a disorder. Such measurements can be carried out. For example, HPLC analysis can be performed on the plasma of animals treated with the drug and the location of radiolabeled compounds can be determined using detection methods such as X-ray, CAT scan and MRI. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by

altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as a model.

Toxicity studies can also be carried out by measuring the blood cell composition. For example, toxicity studies can be carried out in a suitable animal model as follows: 1) the compound is administered to mice (an untreated control mouse should also be used); 2) blood samples are periodically obtained via the tail vein from one mouse in each treatment group; and 3) the samples are analyzed for red and white blood cell counts, blood cell composition and the percent of lymphocytes versus polymorphonuclear cells. A comparison of results for each dosing regime with the controls indicates if toxicity is present.

At the termination of each toxicity study, further studies can be carried out by sacrificing the animals (preferably, in accordance with the American Veterinary Medical Association guidelines Report of the American Veterinary Medical Assoc. Panel on Euthanasia, Journal of American Veterinary Medical Assoc., 202:229-249, 1993). Representative animals from each treatment group can then be examined by gross necropsy for immediate evidence of metastasis, unusual illness or toxicity. Gross abnormalities in tissue are noted and tissues are examined histologically. Compounds causing a reduction in body weight or blood components are less preferred, as are compounds having an adverse effect on major organs. In general, the greater the adverse effect the less preferred the compound.

For the treatment of cancers the expected daily dose of a hydrophobic pharmaceutical agent is between 1 to 500 mg/day, preferably 1 to 250 mg/day, and most preferably 1 to 50 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.

Plasma levels should reflect the potency of the drug. Generally, the more potent the compound the lower the plasma levels necessary to achieve efficacy.

VI. Cell-Based Therapeutics

Cell-based therapeutics rely on the ability of a cell, and in particular a stem cell, to differentiate along a specific cell lineage. The ability to direct lineage-specific differentiation can provide a virtually unlimited suply of source material for the treatment of diseases by tissue repair and regeneration. For example, hematopoetic stem cells have been used for many years to repopulate the bone marrow of animals, including humans, which have lost the ability to produce one or more blood cells. Methods for administering cell-based therapeutics are known to those of ordinary skill in the art. *See, e.g.*, Stein, *et al.*, International Publication No. WO 98/39427, published on March 6, 1997, entitled "Gene Therapy Using Bone Marrow Transplants Transfected With Therapeutic Genes Under the Control of Tissue-Specific Promotors," which is hereby incorporated by reference in its entirety, including all tables, figures, and claims.

EXAMPLES

MATERIALS AND METHODS

Example 1. *In vivo, In vitro*, and Nuclear Transfer Embryos

Cryopreserved bovine *in vivo* embryos were purchased commercially. Bovine cumulus oocyte complexes were recovered from slaughterhouse ovaries by aspiration and *in vitro* matured in maturation medium at 39°C in a 5% CO2 in air atmosphere as described in U.S. Patent No. 5,453,366, issued to Sims *et al.* on September 26, 1995, and/or U.S. Patent No. 5,096,822, issued to Rosenkrans *et al.* on March 17, 1992, each of which is hereby incorporated by reference in its entirety, including all tables, figures, and claims. A preferred maturation medium was prepared by combining 4.4 mL Medium 199 (Gibco-BRL 11150-042), 500 µL fetal calf serum (Hyclone), 50 µL Pen-Strep (Gibco-BRL 15140-122), 50 µL pyruvate (2 mg/mL in medium 199), 25 µL LH (Sioux Biochemical), and 5 µL estradiol (Sigma Chemical E-8875). Matured oocytes were inseminated by combining sperm and matured oocytes in a fertilization drop as described in U.S. Patent Nos. 5,453,366 and/or 5,096,822. CR2 medium (CR1 medium supplemented with amino acids as described in U.S. Patent No. 5,096,822) + 6 mg/mL

was preferred as a fertilization medium. Fertilized oocytes were matured in CR2 medium supplemented with 10% FCS and collected on five day post insemination.

Example 2. Cell Culture Conditions of Donor Cells, Embryonic Germ Cells and Embryonic Stem Cells

Bovine embryonic germ cells were derived from the genital ridge of 55 day old bovine fetuses and cultured in alpha-MEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone) and 0.1 mM 2-mercaptoethanol (Gibco-BRL). Confluent culture dishes were passaged in 1X Trypsin-EDTA (Gibco-BRL) at least once before use in nuclear transfer. Bovine embryonic stem cells were derived from bovine nuclear transfer blastocyst that were on mitotically inactivated mouse fibroblast feeder cells in alpha-MEM (Gibco-BRL). Some ES cell cultures were supplemented with 50 ng/ml recombinant human leukemia inhibitory factor (rhLIF) (R & D Systems), 50 ng/ml fibroblast growth factor basic (bFGF) (R & D systems), and 1X Antibiotic-Antimycotic (Gibco-BRL).

Example 3. Nuclear Transfer Embryos

Matured oocyte complexes were pooled in HECM/HEPES and vortexed for three minutes to strip cumulus and placed in Hoescht medium 30 minutes prior to enucleation. Enucleation (removal of polar body and metaphase plate) was performed and oocytes were flashed with UV light (less than 10 seconds) to confirm enucleation. Enucleated oocytes were washed with HECM/HEPES and put back into a drop of CR2 medium prior to transfer of donor cells within the oocyte cytoplasma. Fusion of the enucleated oocyte and the donor cell was performed on a BTX 200 Electrocell fusion machine in a 500 μM fusion chamber by an electrical pulse of 90 V for about 15 μsec. After fusion the resultant NTs were placed into CR2 medium plus fetal calf serum (Gibco-BRL) until activation. Fused NTs were activated between 4-9 hours later by exposing them to 5 μM ionomycin in HECM/HEPES supplemented with 1 mg/ml BSA for four minutes.

Example 4. RNA Isolation

Total RNA was isolated from single embryos (*in vivo*, *in vitro*, and nuclear transfer) using the RNeasy kit according to manufacturer's protocols (Qiagen). All buffers and reagents were supplied by the manufacturer with the exception of β-mercaptoethanol (Fisher Scientific). Briefly, *in vitro* and nuclear transfer embryos were collected (Day 5) and transfererd into 1.5 ml microcentrifuge tubes containing 350 μL RLT buffer and frozen at –80°C prior to RNA isolation. *In vivo* bovine embryos were cryopreserved prior to RNA isolation and transferred into a 1.5 ml microcentrifuge containing 350 μL RLT buffer prior to RNA isolation. β-mercaptoethanol (0.145M) was added to the RLT buffer and embryos after incubation on ice. The embryos were homogenized by vortexing for 30 seconds. After addition of 70% ethanol (350 μl) the homogenized lysates were applied to the RNeasy mini spin column and centrifuged for 15 seconds at 10,000 rpm (discarded flow-through). The wash buffer RW1 (700μl) was applied to the RNeasy column and centrifuge for 15 seconds at 10,000 rpm (discarded flow-through). The RNA was precipitated by addition of 500 μl of RPE buffer onto the RNeasy column and centrifuged for 15 seconds at 10,000 rpm (discarded flow-through). An additional 500 μl of RPE buffer was applied onto the RNeasy column and centrifuged for two minutes at maximum speed to dry the RNeasy membrane. The RNeasy column was transferred into a new 1.5ml collection tube (supplied by manufacturer) and 30 μl of Rnase-free water was applied directly onto the RNeasy membrane. The RNeasy membrane was centrifuge for one minute at 10,000 rpm to elute the RNA.

Alternatively, RNA is isolated from single embryos using the Micro RNA Isolation Kit (Stratagene) according to the manufacturer's protocols. Briefly, individual embryos were incubated in 200 μL of denaturing buffer and 1.6 μL of β-mercaptoethanol at room temperature for 5 minutes. Extraction was performed in 20 μL of 2M sodium acetate, 200 μL phenol, and 60 μL chloroform:isoamyl alcohol. The aqueous layer was collected and mixed with 1 μL glycogen (10 mg/mL), and precipitated with 200 μL isopropanol. The sample was washed with 70% ethanol, air dried, and resuspended in 16 μL RNase-free water, 2 μL DNase I reaction buffer, 1 μL RNasin, and 1 μL DNase I. The resulting solution was incubated at 37°C for 30 minutes, the nucleic acid was precipitated, and the resulting pellet resuspended in 10 μL DEPC-treated water.

Example 5. First-Strand Synthesis of cDNA and Amplication of cDNA

Total RNA isolated from single *in vivo*, *in vitro*, and nuclear transfer embryos was used to produce cDNA with the SMART PCR cDNA synthesis kit following manufacturer's protocol (Clontech). Briefly, 3 μl of RNA sample was combined with 1μl of cDNA synthesis (CDS) primer (10 μM) (5'-AAGCAGTGGTAACAACGCAGAGTACT$_{(30)}$ N$_{-1}$ N-3'; N=A, C, G, OR T; N1=A, G, or C) and 1 μl of SMART II Oligonucleotide (10μM) (5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3') into a 0.5 ml microcentrifuge tube. Contents were mixed and briefly centrifuged prior to incubation at 70°C in a therma cycler for 2 minutes. After incubation, the tubes were spun briefly in a microcentrifuge to collect contents at the bottom. The tubes were kept at room temperature. The following was added to each reaction tube: 2μl of 5X First-Strand Buffer (250mM Tris-HCl (pH8.3), 375 mM KCl, 30 mM MgC12), 1 μl of DTT (20mM), 1 μl of 50X dNTP (10mM each dNTP), and 1 μl of MMLV reverse transcriptase (Superscript II, 200 units/μl; Gibco-BRL). Microcentrifuge tubes were gently mixed and then spun in a microcentrifuge. The reaction mixtures were overlayed with one drop of mineral oil (to prevent evaporation) and incubated at 42°C for 1 hour in a therma cycler. The first-strand reaction product was diluted by adding 40 μl of TE buffer (10 mM Tris (pH 7.6), 1 mM EDTA). Microcentrifuge tubes were heated at 72°C for 7 minutes to inactivate the reverse transcriptase. For amplication of cDNA, the PCR thermal cycler was preheated to 95°C. For each embryo cDNA sample, 10 μl of single-stranded cDNA was transferred into a 0.5 ml microcentrifuge tube. The following was added to each reaction tube (supplied by manufacturer; Clontech): 74 μl of sterile deionized H_20, 10 μl of 10X Advantage 2 PCR Buffer, 2 μl of 50X dNTP (10mM each dNTP), 2 μl of PCR primer (10 μM) (6FAM-5'-AAGCAGTGGTAACAACGCAGAGT-3'; modified at the 5' end with 6FAM), and 2 μl of 50X Advantage 2 Polymerase Mix. Contents in microcentrifuge tubes were mixed well and spun briefly in microcentrifuge. The reaction mixtures were overlayed with two drops of mineral oil (to prevent evaporation). Thermal cycling paramaters were as follows: one cycle at 95°C for 1 min, followed by 25 cycles at 95°C for 15 sec, 65°C for 30 sec, and 68°C for 6 min. To confirm amplification of

cDNA was successful, a 5 µl aliquot of each sample was electrophoresed on a 1.0% agarose/ethidium bromide gel in 1X TBE buffer. Typical results, indicative of a successful PCR according to the manufacturer (Clontech) had a moderately strong smear of cDNA from 0.5 to 6 kb and several bright bands corresponding to abundant transcripts.

Example 6. Linear Amplification of RNA using T7 polymerase by Reverse Transcription (RT)

10 µL of purified RNA was mixed with 1 µL T7-oligo(dT) primer (5'-TCTAGTCGACGGCCAGTGAATTGTAATAGCACTCACTATAGGGCGT$_{21}$-3') (0.5 mg/mL) to initiate first strand synthesis. The primer and RNA were incubated at 70°C for 10 minutes, followed by incubation at 42°C for 5 minutes. Next, 4 µL of first strand reaction buffer (2 µL 0.1M DTT, 1 µL 10 mM dNTPs, 1 µL RNasin (Promega), and 1 µL SuperScript II (Life Technologies) were added, and the resulting mixture incubated at 42°C for 1 hour. Subsequently, 30 µL of second strand buffer (3 µL 10 mM dNTPs, 4 µL DNA polymerase I, 1 µL *E. coli* RNase H, 1 µL *E. coli* DNA ligase, and 92 µL RNase-free water) was added, and the mixture incubated at 16°C for 2 hours, followed by addition of 2 µL T4 DNA polymerase and incubation at 16°C for 10 minutes. cDNA was extracted with pehnol-chloroform, and washed 3 times with 500 µL on a Microcon-100 column (Millipore).

Amplification was accomplished using the Ampliscribe T7 Transcription Kit (Epicentre Technologies) according to manufacturer's instructions. Briefly, 8 µL of cDNA was added to 2 µL of 10X Ampliscribe T7 buffer, 1.5 µL each of 100 mM ATP, CTP, GTP, and UTP, 2 µL 0.1 M DTT, and 2 µL T7 RNA polymerase, and incubated at 42°C for 3 hours. The resulting RNA was washed 3 times using a Microcon-100 column, collected, and dried to 10 µL.

RNA from the first amplification round was mixed with 1 µL random hexamers (Pharmacia) (1 mg/mL), incubated at 70°C for 10 minutes, chilled on ice, then brought to room temperature. To this sample, 4 µL of first strand buffer, 2 µL 0.1 M DTT, 1 µL 10 mM dNTPs, 1 µL RNasin, and 1 µL SuperScript II were added, and the resulting

mixture incubated at room temperature for 5 minutes, followed by 37°C for 1 hour. Subsequently, 1 μL RNase H was added, followed by an incubation at 37°C for 20 minutes, 95°C for 2 minutes, then chilling on ice. For second strand cDNA synthesis, 1 μL T7-oligo(dT) primer (0.5 mg/mL) was added, and the mixture incubated at 70°C for 5 minutes and 42°C for 10 minutes. To this solution, 30 μL of second strand synthesis buffer, 3 μL 10 mM dNTPs, 4 μL polymerase I, 1 μL *E. coli* RNase H, and 90 μL RNase-free water was added, followed by incubation at 16°C for 2 hours. 2 μL of T4 DNA polymerase was then added, followed by incubation for 10 minutes. Double stranded DNA was then extracted with 150 μL phenol-chloroform, and washed using a Microcon-100 column. The resulting cDNA could be used for a additional rounds of *in vitro* transcription and RNA amplification.

Example 7. Differential Display

The identification of differentially expressed RNAs from *in vivo*, *in vitro*, and nuclear transfer derived embryos was performed using reagents supplies in the Delta Differential Display kit and following manufacturer's protocol (Clontech). Briefly, 2 μl of PCR amplified *in vivo*, *in vitro,* or nuclear transfer single embryo cDNA were used as templates and amplified in the presence of [α-^{33}P]dATP (50nM) (Amersham), 1.0μM each of arbitrary "P" and oligo(dT) primers, 2X KlenTaq PCR reaction buffer, 50 μM each dNTP, and 2.5X Advantage KlenTaq Polymerase Mix. Thermal cycling conditions were as follows: One cycle at 94°C for 5 min, 40°C for 5 min, and 68°C for 5 min; two cycles at 94°C for 2 min, 40°C for 5 min, and 68°C for 5 min; followed by 25 cycles at 94°C 1 min, 60°C 1 min, and 68°C for 2 min; final extension was performed for an additional 7 min at 68°C. Differential display products were loaded onto 6% denaturing polyacrylamide gels and electrophoresed at 70W for a minimum of seven hours. Polyacrylamide gels were transferred to Whatman paper and dried under vacuum at 75°C for 60 min. X-ray film was exposed to the gels at room temperature overnight with intensifying screens. Differentially expressed bands were excised from dried gels and transferred into 0.6-ml microcentrifuge tubes. The gel slices (along with the Whatman paper) were soaked in 100 μl H_2O for 10 minutes, followed by heating at 99.9°C in

thermal cycler for 15 min. Microcentrifuge tubes were spun for 2 min to collect condensation and pellet the gel and paper debris. The supermatants were transferred to new 0.6-ml microcentrifuge tubes and 10 µl of 3M NaOAC, 5 µl of glycogen (10mg/ml) and 450 µl of 100% ethanol were added. Samples were placed at -80°C for 30 min and spun at maximum speed in microcentrifuge for 10 min to pellet DNA. Supernatants were removed and the DNA pellets were washed with 200 µl of ice-cold 85% ethanol. Samples were spun briefly and residual ethanol was removed. DNA pellets were resuspended in 20 µl of sterile H_2O. Eluted bands were stored at -20°C. Differentially expressed bands were reamplified using primer(s) used in the original differential display PCR. Each 12 µl reaction contained 2 µl of eluted DD band, 0.5 µM each primer, 0.8 µM each dNTP, 1.5mM $MgCl_2$, 1X PCR buffer (AmpliTaq) and 0.2 units AmpliTaq DNA Polymerase (Perkin Elmer). Thermal cycling conditions were: 3 min at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension of 4 min at 72°C. Re-amplication products were cloned into pGEM-T vector (Promega) and sequenced using ABI Prism BigDye terminator cycle sequencing kit (PE Applied Biosystems) and automated nucleotide sequencer (GeneSys). The resulting sequencing data were aligned and analyzed using SeqMan (DNASTAR), and BLAST (Basic Local Alignment Search Tool).

Example 8. Embryonic Germ (EG) Cell cDNA Library

A bovine EG cell cDNA library was custom made by Stratagene (La Jolla, CA). Briefly, bovine EG cells isolated from the genital ridges of a slaughterhouse bovine fetus were grown at Infigen, Inc., in α-MEM (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco- BRL) and 0.1 mM β-mercaptoethanol (Gibso BRL). cDNA was synthesized from RNA isolated from 80 x 10^6 EG cells. For directional cloning an Xho I site was introducted at the 3' end of the cDNA, by using an oligo(dT) primer contiaing an Xho I site for priming first strand synthesis, and ligating an EcoRI adapter to the 5' end of the double-stranded cDNA. The directional cDNA was then ligated into lambda arms of the Uni-ZAP vector (Stratagene) cut with EcoRI and XhoI. The average insert size is 1.0 kb with a size range of 0.5-2.2 kb. The estimated amplified titer is 1.2 x 1010 pfy

(plaque forming units)/ml, representing 10^6 recombinants. *In vivo* mass excision of the pBluescript phagemid from the Uni-ZAP XR vector was performed to generate a subtraction library. Briefly, overnight cultures of XL-1Blue MRF' and SOLR cells grown in LB broth supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$ were spun down and resuspended in 10mM $MgSO_4$ to an OD_{600} of 1.0 (8×10^8 cells/ml). In a 50 ml centrifuge tube 10^7 pfu of the amplified lambda bacteriophage library was combined with 10^8 XL1-blue MRF' cells and 10^9 pfu of ExAssist helper phage and incubated at 37°C for 15 minutes. LB broth was added to the mixture and incubated at 37°C for 3 hours with shaking. The centrifuge tube was heated at 65°C for 20 minutes, followed by spining at 1000 x g for 10 min. The supernatant was decanted into a new sterile centrifuge tube, diluted, and combined with 200 µl of SOLR cells (previously diluted to 8×10^8 cells/ml) in a 1.5 ml microcentrifuge tube and incubated at 37°C for 15 min. A portion of the cell mixture was plated onto LB-ampicillin agar plates (100 µg/ml) and incubated overnight at 37°C. Individual colonies were picked from the agar plates and transferred in single wells of a 96 well block containing 1.3 ml LB broth supplemented with 100 µg/ml ampicillian and incubated for 24 hours in a shaking 37°C incubator. The bacterial cells were harvested by centrifugation for 5 min at 1500 x g. Medium was removed by inverting the block. Plasmid DNA was isolated using the R.E.A.L. Prep 96 Plasmid kit (Qiagen) following manufacturer's protocol and supplied reagents. Briefly, bacterial pellets were resuspended in Buffer R2 and lysed after the addition of Buffer R3. The 96 well blocks were placed in a boiling water bath for 5 min and cooled down to room temperature by incubating on ice for 10 min. The bacterial lysates were transferred to the wells of the QIA filter 96 well plate and transferred to another 96 well block by vacuum. The DNA was desalted and concentrated by adding 0.7 volumes of room temperature isopropanol to each well and inverted to mix. The plasmid DNA was pelleted by centrifugation at 2500 x g for 15 min. DNA pellets were washed with 0.5 ml cold 70% ethanol and centrifuged to reconcentrate the pellets. Plasmid DNA pellets were air dried and redissolved in 50 µl of Tris-EDTA, pH 8.0.

Example 9. Sequencing of Bovine EG cDNA/EST Library

Sequencing of cloned cDNA inserts from the EG cDNA library was performed using the ABI Prism Big Dye Terminator cycle Sequencing kit (PE-Biosystems) following manufacturer's protocol and supplied reagents. Sequencing reactions were electrophoresed and analyzed using an automated nucleotide sequencer (Genesys9600 and/or Perkin Elmer ABI 377). The resulting sequencing data were aligned and analyzed using SeqMan (DNASTAR), and BLAST (Basic Local Alignment Search Tools).

Example 10. Macroarray Preparation and Use

Insert cDNA samples from the bovine EG cDNA library were amplified by PCR using flanking vector specific primers T7 and T3. Each 50µl reaction contained 2 µl of DNA template, 1X AmpliTaq Reacton buffer, 1.5 mM $MgCl_2$, .5 µM each primer, 0.8 µM each dNTP, and 0.2 units AmpliTaq DNA Polymerase (Perkin Elmer). Thermal cycling conditions were: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension of 4 min at 72°C. Following PCR amplification of the clone inserts, the PCR products were spotted onto neutral nylon membranes soaked in 0.5 M NaOH/1.5 M NaCl using the HDR tool and Biomek 2000 (Beckman). After spotting onto nylon membranes, the DNA was neutraliszed in 1.0 M Tris-Cl pH 7.4/1.5 M NaCl. DNA was cross-linked by UV irradiation. Nylon membranes were pre-hybridized at 65°C for four hours in modified Church buffer containing 0.25 M Na_2HPO_4 (pH 7.2), 7% SDS, 1mM EDTA and 0.5 mg/ml denatured salmon sperm DNA. The membranes were hybridized in Church Buffer at 65°C for a minimum of 16 hours using ethanol precipitated amplified probe (previously described). Nylon membranes were washed twice in 2X SSC/0.1% SDS at room temperature with gentle agitation. To detect the hybridization of amplified probe to target, the ECF Signal Amplificaiton module (Amersham Pharmacia Biotech) was used. Briefly, after a blocking step, the membrane was incubated with an anti-fluorescein alkaline phosphatase (AP) conjugate (amplified probe contained 6FAM). After washing off the excess conjugate, detection reagent was added and probe-bound AP catalyzed the conversion of the detection reagent to a highly fluorescent product. The fluorescent product was visualized using a signal FluorImager (Molecular Dynamics).

Example 11: Microarray Use

cDNA probes were labeled with Cye-3 and Cye-5 dyes using the Superscript Choice System for cDNA synthesis (Gibco-BRL) according to manufacturer's instructions. DNA affixed on a glass slide microarray was hybridized, and scanned using a Genepix 4000 Scanner and integrated software (Axon Instruments, Inc.). Formamide-based hybridization conditions at 42 °C were preferred over aqueous solutions containing either polyethylene glycol or dextran sulfate. Denhardt's Solution was preferred as a blocking reagent, although SDS, salmon sperm DNA, tRNA, or Cot_1 DNA may be used. Information related to intensity values, intensity ratios, normalization constants, and confidence intervals was assigned to each target. Data was typically viewed as a normalized ratio (Cye-3/Cye-5), in which significant deviations from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression.

Example 12: Developmentally Competent and Developmentally Incompetent Cell Lines

The competence of 59 bovine cell lines previously used in nuclear transfer procedures were compared for the ability to produce live-born animals. The donor cell lines exhibit a range of competencies for successful reprogramming. Critical variables correlated with these competency ranges include culture media, number of passages and days in culture. The minimum standard for competency was defined as producing pregnancy initiation rates of greater than 50%, 90 days gestation, and live birth. 90 days appears to be a key indicator of live birth for cattle, as over 50% of NT fetuses that reach this mark survive full term. In tables 1A and B, embryos generated from a competent donor cell line will be identified by a '+' while - embryos generated from an incompetent donor cell line will be distinguished by a '-'. For example, $BFEG^+$ refers to a competent EG cell line, while $BFEG^-$ refers to an incompetent EG cell line.

Example 13: Assessing the Effect of Changes in a Nuclear Transfer Protocol on Developmental Competence

Two cell lines in particular illustrate the differences in range of competencies: $BFES^+$ and $BFES^-$. These are embryonic stem cell lines cultured under different conditions and used to produce nuclear transfer embryos. Line $BFES^-$ represents a stem

cell line cultured using conditions that produced donor ES cells used for greater than 50,000 nuclear transfers. From this pool of NT embryos, 2000 were transferred into recipients over a two year period, and all failed to develop beyond 55 days in utero. By contrast, using novel culture procedures aimed at minimizing differentiation and maximizing embryonic stem (ES) cell growth, Infigen generated stem cell line $BFES^{+}$ to use as nuclear donors. Briefly, bovine embryonic stem cells were derived from bovine nuclear transfer blastocyst that were on mitotically inactivated mouse fibroblast feeder cells in alpha-MEM (Gibco-BRL). Some ES cell cultures were supplemented with 50 ng/ml recombinant human leukemia inhibitory factor (rhLIF) (R & D Systems), 50 ng/ml fibroblast growth factor basic (bFGF) (R & D systems), and 1X Antibiotic-Antimycotic (Gibco-BRL).

These cells were used to construct embryos that have sustained pregnancies greater than 90 days in 10% of the transferred embryos. This data suggests that developmentally competent reprogramming can be enhanced by culture conditions of donor cell lines.

Example 14: Identifying Molecular Events Related to Developmental Competence by Immunoblot Analysis

Immunoblot analysis was performed using standard protocols and essentially as described in Harlow and Lane (Antibodies: A Laboratroy Manual, pgs 471-506). Briefly, cells were grown as described previously and resuspended in approxiamtely 10 volumes of sample buffer (2% SDS, 100 mM DTt, 60 mM Tris, pH 6.8, 0.1% bromophenol blue). Samples were boiled for 5 minutes and immedialtely loaded onto 10-20% Tris/glycine SDS-polyacrylamide gradient gels. Proteins were separated by electrophoresis at 100-125 V until the dye front reached the bottom of the gel. Proteins were transferred to nitrocellulose in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol) at 100 volts for 1 hour. Mouse anti-histone deacetylase 2 primary antibody (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:100 in blocking solution (5% wt/vol nonfat dry milk, 0.2% Tween 20, 0.02% sodium azide in PBS). Goat anti- mouse secondary antibody conjugated to horse radish peroxidase (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:500, also in blocking solution. Detection

was accomplished using ECL+Western botting detection system (Amershampharmacia, cat. # RPN2132). Immunoblot analysis has identified a potentially novel, 55 kD isoform of bovine histone deacetylase 2 (HDAC2) present in bovine $BFES^-$ donor cells, but absent in competent bovine $BFEG^+$ donor cells. By contrast, an approximately 60 kD band is detected in bovine $BFEG^+$ cells but not $BFES^-$ cells. (Fig. 2).

In addition, we have determined that histone deacetylase 1 is present in bovine $BFEG^+$ cell lines but absent in $BFES^-$ cells (data not shown). It has been presumed that successful reprogramming requires extensive chromatin remodeling, a process highly dependent on histone acetylases and deacetylases. *See, e.g.*, Liang and Pardee, 1992, *Science* **257**: 967-971; Wilmut, 1998, *Scientific American* **279**: 58-63. Taken together, these observations suggest that donor cells can impact reprogramming and developmental competence by activating or deactivating genes and/or biochemical pathways that in turn could enhance or disrupt the reprogramming process. For example, novel deacytalses may alter chromatin remodeling kinetics.

Example 15: Identifying Molecular Events Related to Developmental Competence by Differential Display

Differential display (DD) was used to compare mRNA profiles of single embryos generated by nuclear transfer to *in vivo* embryos. The nuclear transfer embryos were reconstructed from a competent cell line [$BFES^+$] and an incompetent cell line [$BFEG^-$]. DD was used to calculate the percentage of bands conserved between single day 7 *in vivo* embryos, and single day 5 NT embryos generated from incompetent EG^-, and competent ES^+ donor cells. It is important to note that day 7 bovine *in vivo* embryos and day 5 bovine NT embryos have identical morphology, the same number of cells, and are considered to be at the optimal stages for comparison. This analysis revealed a 73% difference in banding patterns between day 7 *in vivo* embryos and day 5 $BFES^+$ embryos, and a 74% difference between day 7 *in vivo* embryos and day 5 $BFEG^-$ NT embryos (Fig 3). A band was considered different if present in the *in vivo* sample but absent in either of the NT samples.

These results suggest that for individual embryos, mRNA expression patterns of embryos reconstructed with donor nuclei may not be converted to blastocyst patterns that represent the best model of successful reprogramming (*i.e.*, *in vivo* produced embryo). These data further suggest a potentially large number of genes may have altered expression levels in NT reconstructed embryos when compared to *in vivo* embryos. The observation that nuclear transfer embryos reconstructed from competent cell lines may also have distinct DD patterns from *in vivo* embryo patterns may partially account for the poor efficiencies of the nuclear transfer process.

By contrast, researchers using differential display protocols demonstrated that expression patterns are highly conserved (~95%) between *in vivo*, IVF, and NT embryos, suggesting that developmental programs very similar to those detected for *in vivo* embryos can be established after nuclear transfer. *See* DeSousa *et al.*, 1999, *Cloning* 1: 63-69. However, this analysis was based on single embryo equivalent representations obtained from pools of embryos. This method can mask differences between individual embryos, which in turn may account for individual embryo differences during development and the low live birth rates observed by artisans. For example, if 20% of NT embryos were developmentally competent, pooling template from 5 embryos likely would produce results substantially similar to results from a competent *in vivo* embryo. The data provided herein suggest that the vast majority of individual, NT reconstructed embryos may not reproduce expression patterns similar to *in vivo* patterns. The ability to monitor single embryos is critical to minimize genetic noise that might obscure underlying reproducible expression patterns. Since differences appear to be readily detectable at a single embryo level, deficiencies and/or differences in the mRNA profiles of NT embryos when compared to *in vivo* embryos ultimately will help identify genes/mechanisms responsible for low (live birth) efficiencies and developmental problems.

Figure 4 describes comparing banding patterns generated by differential display (Figure 4A & B) between five individual day 7 *in vivo* embryos (lanes 1-5); six individual day 5 IVF embryos (lanes 6 and 11)]; five individual embryos reconstructed by NT [three day 5 embryos (lanes 12-14), one day 7 (lane 15) and one day 8 (Lane 16)]

using a developmentally incompetent cell line, and the developmentally incompetent donor cell (DC) line . Day 7 bovine *in vivo* embryos and day 5 bovine NT embryos have identical morphology, the same number of cells, and are considered to be at the proper stages for accurate comparison. Briefly, RNA from each embryo was isolated, reverse transcribed and amplified using the protocol described previously. Differential display reactions were performed using one of 15 primer pairs. Analysis of banding patterns (Fig. 4A) revealed 122 bands present in all 5 individual day 7 in vivo embryo samples. Seventy three (60%) of the 122 bands were also identified in five day 5 individual IVF produced embryos. (Less than 10% of the bands were present in the sixth, day 6 IVF embryo, lane11.) In sharp contrast only 9 (7%) of the 122 bands were identified in all of the day 5 individual embryos reconstructed by NT (Fig. 4B). (The number of matching bands was less for day 7 and day 8. The bands that did match showed altered expression patterns.) Moreover, these were the only 9 bands (of over 700 identified bands observed cumulatively in the 5 NT samples) present in all day 3 NT reconstructed embryos. This indicates tremendous heterogeneity among individual NT produced embryos collected at precise times after activation and not observed in either in vivo or IVF embryos. These banding patterns further support the hypothesis that low NT efficiencies may be due partly to improper reprogramming, exemplified by the different banding patterns between individual embryos reconstructed by NT. Most importantly, these data indicate that genes expressed at high levels in developmentally competent embryos, but at low or undetectable levels in developmentally incompetent embryos can be identified and used to determine an idealized expression pattern. The consistent amplification of identical bands in all individual *in vivo* embryo samples and all individual IVF embryo samples further demonstrates that the embryo harvesting and amplification protocols described herein are reliable for detecting gene products whose expression levels are either relatively high or low.

These results also suggest that, for individual embryos, mRNA expression patterns of embryos reconstructed with donor nuclei may not be converted to blastocyst patterns that represent a best model of successful reprogramming (i.e., *in vivo* produced embryo). This is further supported in a report by Eckert and Niemann (Mol. Human Reprod. 4: 957-65, 1998) who identified perturbations in mRNA expression patterns

specific to the LIF-LIF receptor system in embryos generated *in vitro* and possibly correlated with improper blastocyst development. By contrast, de Sousa *et. al.* used differential display protocols to demonstrate that expression patterns are highly conserved (~95%) between *in vivo*, IVF, and NT embryos, suggesting that developmental programs very similar to those detected for *in vivo* embryos can be established after nuclear transfer. However, their analysis was based on pools of embryos. This method can mask differences between individual embryos, which in turn may account for individual embryo differences during development and the low live birth rates observed . For example, if 20% of NT embryos were developmentally competent, pooling template from 5 embryos likely would produce results substantially similar to results from a competent *in vivo* embryo. The data provided herein suggests the vast majority of individual, NT reconstructed embryos may not reproduce expression patterns similar to *in vivo* patterns. The ability to monitor single embryos is critical to minimize genetic noise that might obscure underlying reproducible expression patterns. Since differences appear to be readily detectable at a single embryo level, deficiencies and/or differences in the mRNA profiles of NT embryos when compared to *in vivo* embryos may help identify genes/mechanisms responsible for low (live birth) efficiencies and developmental problems.

Example 16: Identifying Molecular Events Related to Developmental Competence by Differential Display Using Microarrays

Though differential display can be used to identify reprogramming differences between embryos generated by NT and those produced *in vivo*, the method cannot be used in a high throughput format and cannot be performed on a sufficiently broad scale to characterize reprogramming at a molecular level. Limitations include very labor intensive procedures after identification of differentially expressed bands and confirmation of differential expression. Also, standard differential display does not allow genomic scale comparison and sophisticated statistical analysis of expression data, and thus prevents 'rapid' elucidation of comprehensive molecular patterns and relationships. To reconfirm our results and compensate for the limitations of differential display cDNA microarray technology was used to investigate and compare expression profiles of single *in vivo* and *in vitro* derived embryos (Fig. 5).

The microarray was comprised of cDNA clones representing numerous functional classes and gene families, including unknown ESTs, genes putatively associated with reprogramming (SNF2), cell cycle progression (quiescen, cyclins), cell adhesion-extracellular matrix (collagen, fibronectin), apoptosis (p53), imprinting (Igf2 and Igf2r), transcription (STAT), embryonic signaling (interferon tau), and signal transduction (JAK) (Fig. 6).

To amplify and detect hybridization signals, protocols for linear and exponential amplification of cDNA representing mRNA from a single embryo were employed. *See, e.g.*, Van Gelder *et al.*, U.S. Patent No. 5,716,785, issued on February 10, 1998, which is hereby incorporated by reference, including all tables, figures, and claims. After incorporating appropriate controls 744 PCR amplified arrayed cDNA clones obtained from an arrayed EG cell cDNA library were spotted onto nylon membranes, which in turn were probed with cDNA representations of a single *in vivo* embryo and 4 single embryos generated by NT from 1 competent (+), 1 unknown, and 2 incompetent (-) cell lines. A similar comparison of conservation after hybridization has confirmed basic plus/minus differences in expression patterns between individual embryos. Embryos generated from the 2 incompetent and unknown cell lines ranged in similarity from 18-85%, while the embryo generated from the competent cell line had a similarity to the *in vivo* embryo of 88%. Even a 3% difference in similarity between NT embryos generated from competent and incompetent (+ and -) donor cell lines represents ~22 of the 744 genes screened with detectable (on/off) plus/minus differences. Studies in Phase II propose measuring expression levels of 10,000 genes. These data suggest that, from this pool, as many as 300 genes may have detectable plus/minus expression levels, and many more will likely have less subtle, but measurable differences. These results are the first visualization of broad changes in mRNA expression patterns between individual nuclear transfer and *in vivo* embryos.

Nucleotide sequences analyzed by the methods described herein are provided in Tables 2 and 3. Each sequence was determined to have a positive, negative, or neutral association with successful cellular reprogramming. The individual nucleotides in these sequences are provided as A=adenine; T=thymine; G=guanine; C=cytosine;

N=nucleotide not determined. Individual sequences in Table 2 begin with a sequence identifier, and are separated by blank lines, while those in Table 3 are separated by two blank lines.

Example 17: Statistical Analysis of Molecular Events Related to Developmental Competence

Clustering analysis was used to identify an idealized expression pattern for a developmentally competent embryo. 14 genes uniquely associated with reprogramming were identified (Fig. 7). The following EST sequences were identified as being associated with reprogramming: 990809a-88, 990726a-13, 990726a-14, 990726a-14, 990729a-1, 990729a-13, 990928a-9, 990928a-10, 990928a-65, 991108a-13, 991108a-14, 991108a-87, 991115a2-13, 991115a2-24, and 991115a2-92. Thirteen genes were always expressed in both the *in vivo* and competent cell derived embryos, but not in any of the embryos generated from incompetent cell lines. One gene was not expressed in the 2 competent embryo samples, but was detected in all three incompetent samples. The embryo derived from the unknown cell line had an expression pattern that matched 100% the embryos generated from the 2 incompetent cell lines. Transfer of embryos generated from the unknown cell line into recipient heifers failed to meet the criteria of a developmentally competent cell line (no pregnancy initiation was detected for any of the recipients), suggesting that it may be feasible to 1) identify an 'idealized expression pattern' of genes representing developmentally competent reprogramming and 2) identify genes that can be used to predict developmental viability. Gene expression differences between *in vivo* and nuclear transfer embryos are likely to contribute to the high inefficiencies associated with nuclear transfer cloning and potentially represent reprogramming deficiencies.

Example 18: Identifying Developmentally Competent and Incompetent Nuclear Donor Cell Lines, and Developmentally Competent and Incompetent Nuclear Transfer Embryos

In order to determine if a nuclear donor cell line is comprised of developmentally competent or incompetent cells, one or more cells are separated from the cell line and used as nuclear donors to provide one or more nuclear transfer embryos by the methods described herein. RNA or protein is isolated, and optionally amplified, for identification

of molecular markers that indicate developmental competence or incompetence. If the embryos are cultured *in vivo* or *in vitro* to at least the two cell stage, the embryo can be divided into two or more portions, such that at least part of the embryo is retained for possible implantation into a maternal host.

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The invention illustratively described herein may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

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